Biology of Extracellular Matrix 9

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Karin Forsberg-Nilsson *Editors*

Proteoglycans in Stem Cells

From Development to Cancer

Biology of Extracellular Matrix

Volume 9

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Extracellular matrix (ECM) biology, which includes the functional complexities of ECM molecules, is an important area of cell biology. Individual ECM protein components are unique in terms of their structure, composition and function, and each class of ECM macromolecule is designed to interact with other macromolecules to produce the unique physical and signaling properties that support tissue structure and function. ECM ties everything together into a dynamic biomaterial that provides strength and elasticity, interacts with cell-surface receptors, and controls the availability of growth factors. Topics in this series include cellular differentiation, tissue development and tissue remodeling. Each volume provides an in-depth overview of a particular topic, and offers a reliable source of information for post-graduates and researchers alike.

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Preface

Over the last few decades, stem cell research has taken center stage in the attention of researchers and the public, not least with the awarding of the Nobel Prize for Physiology and Medicine to John B. Gurdon and Shinya Yamanaka in 2012 for their discovery that mature cells can be reprogrammed to become pluripotent (induced pluripotent stem cells, iPSCs). Indeed, stem cells have tremendous potential in research, allowing the generation of various human tissue-specific cell types and lately also organoids. These human research tools were previously impossible to obtain, both in purity and at scale, but based on embryonic stem cells (ES cells) and later iPSC technology, they now serve as useful model systems for basic and disease-oriented research. In the biomedical field, stem cells and aberrant stem cell function have been identified as culprits for disease progression and therapeutic resistance and have therefore become important biomarkers as well as therapeutic targets.

Under physiological conditions, stem cells are characterized by defined properties such as asymmetrical cell division, unlimited proliferative potential, and pluri- or multipotency. These stem cell criteria are in accordance with their role to generate daughter cells which—after a series of differentiation steps—have the ultimate goal of forming a cell type which fulfills a defined function in a multicellular organism, such as a muscle cell, a leukocyte, or a nerve cell. Much has been learned about stem cell function through the study of developmental processes in model organisms. This research has allowed us to identify defined signaling pathways which—via specific interactions of soluble or cell surface-bound ligands with receptors on target cells—convey signals to cells which either promote or inhibit cell differentiation. Indeed, these signals determine if a stem cell remains in a quiescent state, or if it proceeds to differentiate into a specific cell type. Notably, the research efforts of numerous laboratories have revealed that stem cell function is not solely dependent on (stem) cell-autonomous properties and their interaction with growth factors, morphogens, and juxtacrine signaling molecules but also on the extracellular matrix (ECM) within the so-called stem cell niche.

ECM is a complex macromolecular meshwork of structural glycoproteins, proteoglycans, and glycosaminoglycans, adaptor proteins linking these constituents,

and a plethora of functional ligands bound to them. Matrix composition is not only determined by different rates of biosynthesis of individual components but also subject to modification and degradation by specific proteases and glycosaminoglycan-degrading and editing enzymes. Besides providing a structural framework that determines the architectural and mechanical properties of a given tissue, the constituents of the ECM have an essential function in modulating signaling processes with particular relevance to stem cell function. ECM components play pivotal roles by either enhancing or inhibiting the function of ligand–receptor pairs in numerous signaling events, including the WNT, NOTCH, Hedgehog, and FGF-signaling pathways. Moreover, they contribute to proper morphogen gradient formation during development, which adds to the complexity of developmental signaling in cases where an optimal ligand concentration is required to drive a carefully orchestrated morphogenetic process.

We have chosen to focus on proteoglycans and glycosaminoglycans owing to their importance in modulating stemness-associated signaling processes. Glycosaminoglycans are highly negatively charged carbohydrates composed of repetitive disaccharide units which can be attached to core proteins, thus forming proteoglycans. As discussed in this book, heparan sulfate (HS) is a structurally diverse type of sulfated glycosaminoglycan that is ubiquitously expressed on the cell surface and in the extracellular matrix where it interacts with numerous growth factors and morphogens. The association of HS chains with various protein partners is surprisingly unique and dictated by the spatiotemporal expression of various modifying enzymes. Our book highlights the role of specific HS structures generated by different sets of biosynthetic enzymes, and of specific HS proteoglycans of the syndecan and additional proteoglycan families in modulating and orchestrating signaling in the stem cell niche, thus modulating the quantity, quality, and activity of stem cells during development, aging, and disease. Likewise, the role of specific sulfation patterns of the glycosaminoglycan chondroitin sulfate (CS) and of specific CS proteoglycans such as NG2/CSPG4 and versican in development and tissue homeostasis as well as malignant disease will be presented. Furthermore, we will highlight the function of the non-sulfated glycosaminoglycan hyaluronan, and its receptor CD44, a single-chain transmembrane glycoprotein able to modulate several mechanisms that control stem cell behavior, including migration and anchorage, efflux of toxic compounds, growth under hypoxic conditions, and quiescence, besides the properties of self-renewal and differentiation potential. The function of proteoglycans and glycosaminoglycans will be integrated into their interaction with other ECM compounds in this context, including large matrix glycoproteins such as tenascins, enzymes modulating their function such as proteases of the ADAMTS family, and glycosaminoglycan-degrading enzymes such as heparanase and hyaluronidases.

Written by leading experts in the field, the chapters of this book provide a comprehensive overview of the current knowledge on the role of proteoglycans and glycosaminoglycans in stem cell function in physiological processes and disease. Besides describing their role in development, developmental disorders, and physiological functions of embryonic and adult stem cells, several chapters focus on their pivotal role in aberrant stem cell function during neurodegenerative and

malignant disease, thus focusing on major widespread diseases. Indeed, several chapters address the topic of tumor-initiating cells ("cancer stem cells, CSCs"), which are considered a key tumor subpopulation with stem-cell-like properties that may give rise to tumor relapse due to stemness-associated properties such as unlimited proliferative potential, high developmental plasticity, and increased resistance to chemo- and radiotherapy.

The knowledge on ECM function in the stem cell niches and cancer stem cell niches presented in this book comprises a wide range of experimental systems. They range from biochemical and cell biological studies, in vitro studies on embryonic stem cells, induced pluripotent stem cells, and adult stem cells, model organisms including the fruit fly *Drosophila melanogaster*, the zebrafish model, and genetically altered mice as well as pathological studies on human tissues and clinical trials in humans, thus providing a complete view on the topic. Moreover, we will present translational data that mark several ECM constituents of the stem cell niche as an important therapeutic target for malignant, neurodegenerative, and other diseases.

As editors, we hope you will enjoy this new issue of the *Biology of Extracellular Matrix* series, as we believe that the information in this volume will be useful for the research community about the broad range of ECM functions associated with stem cell function in the context of development and disease. We expect that the acquisition of a deeper knowledge in this rapidly evolving field will stimulate new developments in basic, applied, and clinical aspects of stem cell research.

Martin Götte
Karin Forsberg-Nilsson

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Chapter 1
Heparan Sulfate Proteoglycans in the Stem Cell Niche: Lessons from *Drosophila*

Nanako Bowden and Hiroshi Nakato

Abstract In addition to the obvious importance of stem cells in regenerative medicine, their unique behaviors and ingenious molecular systems for their control are of particular interest in the context of basic cell biology. *Drosophila* genetics plays a pivotal role to reveal fundamental principles of stem cell control mechanisms. Namely, *in vivo* studies using lineage tracing techniques elucidated the cellular and molecular mechanisms of the interactions between stem cells and the extracellular microenvironment. As one of the key components of the stem cell niche, heparan sulfate proteoglycans (HSPGs) have critical functions in regulating stem cell behavior. HSPGs serve as co-receptors for numerous ligands such as fibroblast growth factors, bone morphogenetic proteins, Wnt-related factors, hedgehog, and cytokines, which are all imperative regulators of stem cell behaviors. By modulating and orchestrating these niche factors' signaling and distribution, HSPGs control the quantity, quality, and activity of stem cells.

Abbreviations

BMP	bone morphogenetic protein
CySC	cyst stem cell
Dally	Division abnormally delayed
Dlp	Dally-like protein
Dpp	Decapentaplegic
FSC	follicle stem cell
GSC	germline stem cell
Hh	Hedgehog
Hs6st	HS 6-O sulfotransferases
HSPG	heparan sulfate proteoglycan

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ISC	intestinal stem cell
NDST	HS *N*-deacetylase/*N*-sulfotransferase
Sfl	sulfateless
Sulf	HS 6-*O* endosulfatase
Trol	Terribly reduced optic lobe
Upd	Unpaired
Wg	Wingless

1.1 Introduction

Stem cells are maintained in specialized microenvironments called "niches," which support their stemness (Spradling et al. 2001). The niche is usually composed of niche cells, extracellular matrix components, and signaling factors emanated from the niche cells. It is poorly understood how each niche factor affects stem cell behavior. For the future development of novel stem cell-based therapies in regenerative medicine as well as drug discoveries for cancers, it is critical to define the molecular mechanisms by which the niche controls stem cell maintenance and differentiation.

The *Drosophila* genetics offers a powerful model system in stem cell biology and has led to the understanding of basic principles of stem cell control. *In vivo* studies using this model have highlighted the importance of various sets of molecules in the stem cell niche. For example, heparan sulfate proteoglycans (HSPGs) were found to be universal niche factors that play critical roles in controlling stem cell behavior (Nakato and Li 2016). HSPGs are a unique class of carbohydrate-modified proteins required for normal cell-to-cell communications. HSPGs serve as co-receptors for a wide array of signaling ligands on the cell surface. Such signaling molecules that require HSPG co-receptors for proper signaling, or HS-dependent factors, include fibroblast growth factors, bone morphogenetic proteins (BMPs), Wnt-related proteins, Hedgehog (Hh), and ligands of the Jak/Stat and EGFR pathways (Nakato and Li 2016). HSPGs generally regulate the activity of signaling receptors in a cell autonomous manner. In some contexts, however, HS expressed by adjacent cells can reach the surface of the neighboring signal receiving cells and affect signaling *in trans* ("*trans*" co-receptor activity) (Jakobsson et al. 2006; Hayashi et al. 2009; Dejima et al. 2011). Additionally, HSPGs control a range and patterns of diffusion of the abovementioned signaling ligands in a tissue (Fujise et al. 2003).

HSPG function is largely controlled by the fine structure of HS chains. HS biosynthesis involves sequential modification events, initiated by *N*-deacetylation and *N*-sulfation of glucosamine residues catalyzed by HS *N*-deacetylase/*N*-sulfotransferase (NDST). This reaction is requisite for subsequent HS modifications, including *C*-5 epimerization of glucuronic acid residues and *O*-sulfation at various ring positions. Following these HS modification steps in the Golgi apparatus, extracellular HS 6-*O* endosulfatases (Sulfs) further modify HS structure on the cell

surface (Dhoot et al. 2001; Morimoto-Tomita et al. 2002; Kleinschmit et al. 2010, 2013). Sulfs specifically cleave 6-*O* sulfate groups on HS. These biosynthetic (by the Golgi enzymes) and post-biosynthetic (by Sulfs) events determine the number of ligand-binding sites on HS as well as the affinity of HS for various ligands. This enables HSPGs to quantitatively regulate signaling output by these factors.

Drosophila has several evolutionarily conserved classes of HSPGs: the syndecan (Sdc), two glypicans called Division abnormally delayed (Dally) and Dally-like protein (Dlp), the perlecan, Terribly reduced optic lobe (Trol), and a new member of the testican family, Carrier of Wingless (Cow). The *Drosophila* model has several unique advantages in proteoglycan biology (Nakato and Li 2016). First, *Drosophila* has all major HSPG core proteins and a complete set of HS biosynthetic/modifying enzymes (Kamimura et al. 2001, 2006), and the complex structural features of mammalian and *Drosophila* HS chains are comparable (Nakato et al. 2019). Second, there is no genetic redundancy in *Drosophila* genes for the HS biosynthetic machinery. This simplifies the interpretation of the genetic analysis of HS functions. Finally, the availability of numerous genetic tools enables us to manipulate HSPG structure and function *in vivo* with a single-cell resolution (Kamimura et al. 2011).

Remarkably, most niche factors thus far identified in different stem cell models are HS-dependent signaling molecules, such as BMPs, Wnts, Hh, and Unpaired (Upd), a ligand of the *Drosophila* Jak/Stat pathway (Hayashi et al. 2012). This fact had suggested that HSPGs may play key roles in stem cell control. Indeed, this was the case. Recent studies established HSPGs as an evolutionarily conserved, universal, and essential niche component, which controls various aspects of stem cell behaviors in many organs through different mechanisms. This chapter focuses on a few genetic studies of *Drosophila* stem cells that approached fundamental problems in stem cell biology: how are the quantity, quality, and activity of stem cells precisely controlled in the niche?

1.2 Heparan Sulfate Proteoglycans Regulate Stem Cell Number in the *Drosophila* Germline Stem Cell Niche

1.2.1 The Drosophila *Female Germline Stem Cell Niche in the Ovary*

The *Drosophila* ovary is the largest organ in the female abdomen with a polarized structure. It contains germline stem cells (GSCs) at the anterior edge, mature oocytes at the posterior end, and progressively developing germline cells in between (Fig. 1.1a). Two or three GSCs are found at the anterior tip of a structure called germarium. The chief component of the GSC niche is a group of somatic cells, the cap cells, which regulate GSC maintenance in a contact-dependent manner (Fig. 1.1b).

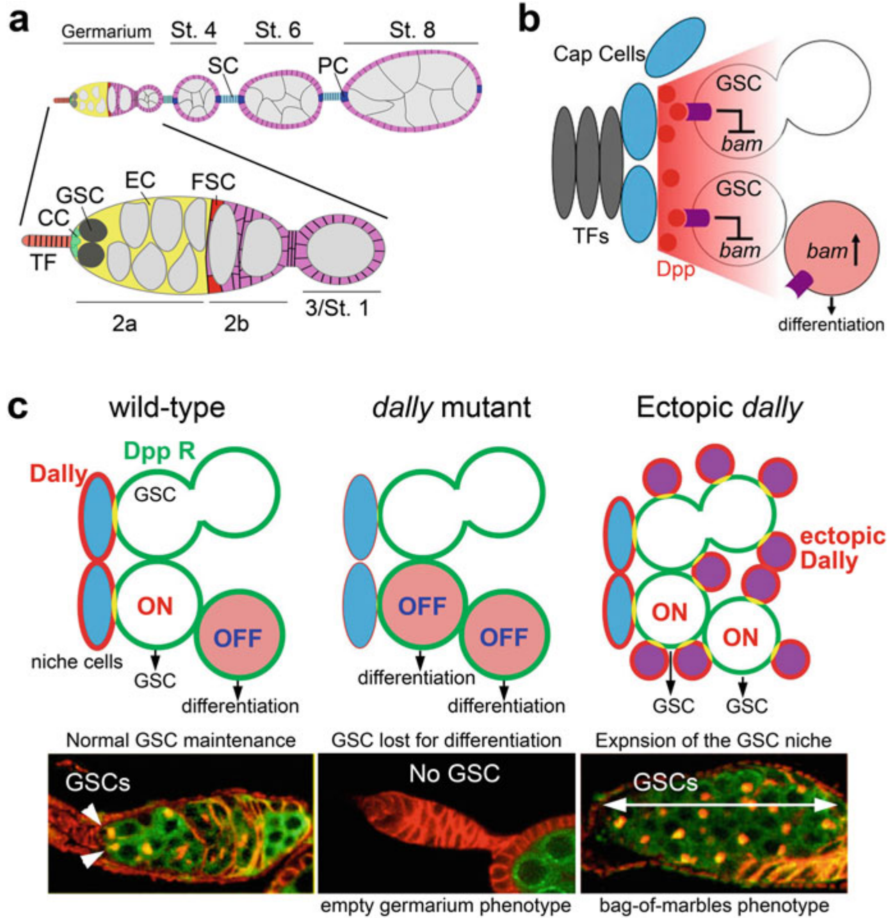

Fig. 1.1 HSPGs in the *Drosophila* female germline stem cell niche. (**a**) The *Drosophila* ovariole. The *Drosophila* ovary is composed of strings of progressively developing egg chambers. Each string is called ovariole (top), which contains a structure called the germarium at its anterior edge (bottom). The germarium contains both GSC (dark gray) and FSCs (red). Different types of somatic cells surround the germline cells: terminal filament cells (TF, orange), cap cells (CC, green), follicle cells (pink), polar cells (PC, blue), and escort cells (EC, yellow). Differentiating germ cells are shown in light gray, and the developmental stages of germ cells are also indicated. Anterior to the left; posterior to the right. (**b**) The GSC niche. When a GSC divides, two daughter cells are produced in a way that one daughter keeps a contact with the cap cells (blue) and the other dissociated from them. The cap cells secrete Dpp (red circle), which represses *bam* only in the contacting GSC daughter. This cell remains a GSC. In the other daughter cell, *bam* directs differentiation into female germ cells. Thus, Dpp regulates GSC fate in a contact-dependent manner. (**c**) Model for the "contact-dependent" regulation of the GSC niche. (left) A dividing GSC (top) produces two daughter cells (bottom). In wild-type, Dally (red) is expressed on the cell surface of the cap cells (blue). Dpp receptors (green) are expressed on germ cells. Dpp activates its receptors at the interface of two contacting cells where receptors and co-receptor "meet" (yellow). Therefore, Dpp signaling is activated in the contacting GSC daughter (ON) but not in the detached daughter (OFF). (middle) In the absence of *dally*, Dpp signaling is impaired. GSC daughters, even when they contact with the cap cells, differentiate, and therefore stem cell populations are lost. This results in the loss of germ cells in a germarium (empty germarium phenotype). (right) Ectopically expressed

The primary molecule that regulates GSC maintenance in the ovary is Decapentaplegic (Dpp), a fly homologue of the BMP2/4 (Fig. 1.1b; Xie and Spradling 1998). Dpp was the first identified niche factor in any stem cell models in all species. Dpp is a highly pleiotropic molecule: it plays critical roles in morphogenesis of many organs throughout development. For example, Dpp acts as a long-range morphogen and regulates patterning along the anterior–posterior axis of the developing wing. In the ovary, Dpp is secreted from the cap cells and activates downstream signaling only in GSCs that directly contact the cap cells. In these cells, Dpp signaling represses expression of *bag-of-marbles* (*bam*). *bam* encodes a translation repressor which acts as a GSC differentiation factor: it is both necessary and sufficient for GSC differentiation (Shen et al. 2009). Since Dpp signaling is activated in a contact-dependent fashion, *bam* is expressed in GSC daughter cells, which have lost contact with the cap cells. These cells differentiate to produce oocytes and nurse cells.

1.2.2 Dally Regulates Stem Cell Number in the GSC Niche

The function of Dpp in the female GSC niche demonstrated that the contact-dependent control system of stem cell fate decision works elegantly in small niches like ones for the *Drosophila* GSCs. However, one crucial question remained unanswered: how can Dpp work in a contact-dependent fashion? Since Dpp is an extensively analyzed, well-known long-range morphogen, it had been a mystery why this molecule cannot act in the non-contacting GSC daughter, which is only a single-cell diameter away from the source of Dpp production.

Dally, a *Drosophila* glypican, was found to be a key player in this regulation (Hayashi et al. 2009; Guo and Wang 2009). In the developing wing imaginal disc, Dally serves as a Dpp co-receptor (Fujise et al. 2003; Akiyama et al. 2008; Belenkaya et al. 2004). In the GSC niche, on the other hand, Dally acts as a "*trans*" co-receptor (Hayashi et al. 2009; Dejima et al. 2011). Dally is specifically expressed on the surface of the cap cells and mediates Dpp signaling *in trans* in a directly contacting GSC (Fig. 1.1c left). In the absence of *dally*, Dpp signaling in these GSCs is compromised. As a result, GSCs are lost to differentiation. As shown in Fig. 1.1c (middle), *dally* mutants show the "empty germarium" phenotype in which germ cells are lost from the germarium. In contrast, when *dally* is overexpressed in somatic cells outside the niche, Dpp signaling is ectopically activated in an expanded area (Fig. 1.1c right). Consequently, GSC-like cells

Fig. 1.1 (continued) dally outside of the niche induces ectopic activation of Dpp signaling throughout the germarium (yellow). Germ cells are maintained as actively dividing "stem cell-like" cells, leading to germline tumors (bag-of-marbles phenotype). The figures are modified from the following publications: Fig. 1.1a (Su et al. 2018) with permission from the Genetics Society of America; Fig. 1.1c (Hayashi et al. 2009) with permission from the Rockefeller University Press

actively divide and expand, resulting in a swollen germarium. This phenocopies the "bag-of-marbles" phenotype caused by loss of the differentiation factor, *bam*.

This ability of Dally to act as a Dpp *trans* co-receptor explains the contact-dependent control of GSC fate decision. Dpp receptors on the surface of GSCs and Dally co-receptor expressed on the cap cell surface can "meet" only at the GSC/cap cell interface, where Dpp activates its receptors. Thus, HSPG *trans* co-receptors act as a key determinant of stem cell number.

1.2.3 *The* Drosophila *Male Germline Stem Cell Niche in the Testis*

"Asymmetric division," which can produce both differentiating cells and stem cell daughters, is a key feature of stem cells. In some stem cell systems, the asymmetric division is tightly regulated; daughter cells are produced by a precisely oriented division in a way that one daughter cell is kept in the niche while the other daughter is displaced (Yamashita et al. 2010). Such a system is employed in the male GSC niche in the testis (Yamashita and Fuller 2008; Yamashita et al. 2010).

The testis contains two types of stem cells: GSCs and cyst stem cells (CySCs) at its apical tip. CySCs are stem cells for somatic cell populations, and a pair of CySCs enwrap each GSC. A group of somatic cells called the hub function as the niche for both GSCs and CySCs. Like the female GSC niche, the maintenance of stem cells is dependent on the contiguity with the hub. In GSCs, centrosomes are anchored at the hub interface (Fig. 1.2a; Yamashita et al. 2003). A protein complex called the "centrosome anchoring machinery" regulates proper centrosome positioning at the GSC cortex adjacent to the hub (Yamashita et al. 2003; Inaba et al. 2015). In a subsequent step, the daughter centrosome migrates to the opposite side of the cell while the mother centrosome remains in the original position (Fig. 1.2a). This establishes the spindle axis perpendicular to the hub interface and supports the stereotypical asymmetric division of GSCs. Failure in proper anchoring of the mother centrosome near the hub leads to uncontrolled division orientation, which in turn causes aberrant stem cell numbers.

1.2.4 *HS in the Niche Regulates GSC Asymmetric Division*

Sulfateless (Sfl) is the only NDST gene in *Drosophila*, responsible for the first step of HS modification. Since *N*-sulfation is essential for all subsequent HS modification reactions, loss of *sfl* disrupts biological activities of HS chains (Lin and Perrimon 1999; Lin et al. 1999). Blocking HS biosynthesis by *sfl* RNAi knockdown specifically in the hub of the testes leads to an increase in the number of GSCs. The increased number of GSCs is associated with centrosome mispositioning, leading to

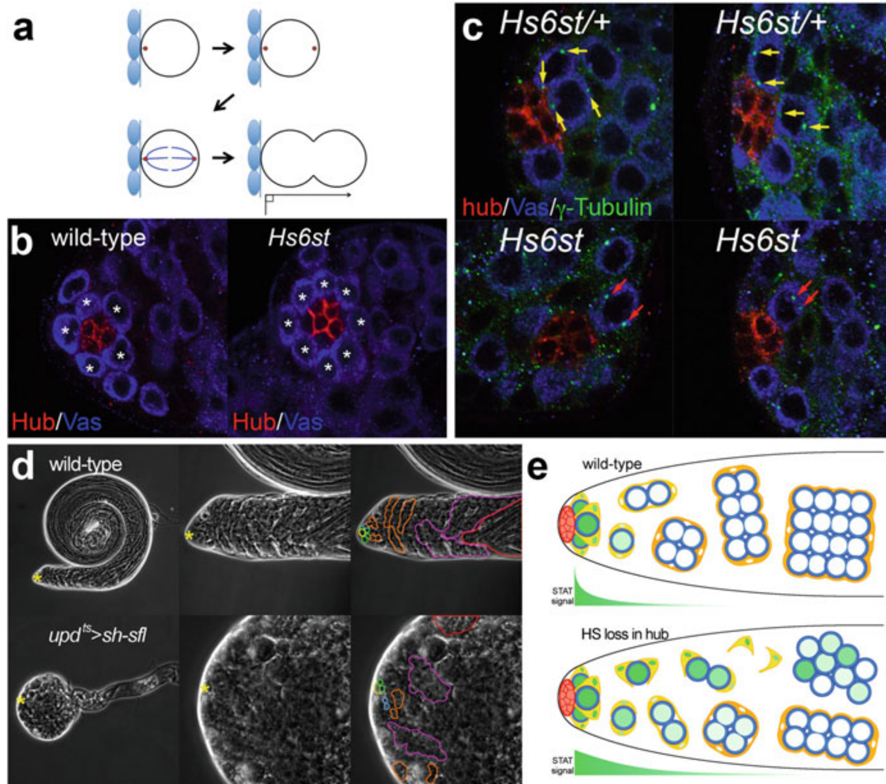

Fig. 1.2 HSPGs in the *Drosophila* male germline stem cell niche. (**a**) In male GSCs, the centrosome anchoring machinery anchors the mother centrosome of a GSC (red dot) near the hub (light blue). The daughter centrosome migrates to the opposite side of the cell, which establishes the orientation of the spindles (blue) in perpendicular to the hub interface. (**b**) HS 6-*O* sulfation regulates GSC number. GSCs (asterisks) are shown for wild-type (left) and *Hs6st* mutant (right) testes. Hub and germ cells are stained with anti-E-cadherin (red) and anti-Vasa (blue) antibodies, respectively. (**c**) *Hs6st* mutation perturbs centrosome positioning in GSCs. Pairs of properly oriented centrosomes (yellow arrows) are shown in *Hs6st/+* testes (top). In *Hs6st* mutants (bottom), the mispositioning of centrosomes (red arrows) is observed. Centrosomes are stained with anti-gamma-tubulin antibody (green). (**d**) Roles of hub HS in the organization of spermatogenic cells. Wild-type (top) and hub-specific *sfl* knockdown (bottom) testes are shown. Two right images are high magnification views of the left image in each row. Different cell types are marked as follows: yellow, hub; green, GSCs; blue, gonialblasts; orange, spermatogonia; pink, primary spermatocytes; red, elongating spermatids. In wild-type, ordered localization of the progressive stages of spermatogenesis is observed. In the absence of hub HS, a severe "tumorous" phenotype with disorganized spermatogenic patterns is observed. Yellow asterisks indicate the hub. (**e**) Model for the role of hub HS in the range of Jak/Stat signaling. CySCs and GSCs are shown in yellow and blue, respectively. (top) In wild-type, HS sequesters Upd ligand at the hub. Sufficient Jak/Stat signaling (green) is activated in stem cells directly contacting the hub. (bottom) In the absence of hub HS, the range with high Jak/Stat signaling is expanded. This produces ectopic populations of stem cells at distant locations from the niche. The figures are modified from the following publications: Fig. 1.2b and c (Levings et al. 2016) with permission from the American Society for Cell Biology; Fig. 1.2d and e (Levings and Nakato 2018) with permission from Oxford University Press

spindle misorientation and symmetric GSC division (Levings et al. 2016). Therefore, besides mediating niche factor signaling, HS expressed in the niche has another role; it regulates GSC number non-cell autonomously by controlling centrosome positioning and spindle orientation.

The 6-*O* sulfate group of glucosamine residues is a key component of the binding sites on HS for many protein ligands both in mammals and in *Drosophila* (Ai et al. 2003; Kamimura et al. 2001, 2006; Kleinschmit et al. 2010, 2013; Wojcinski et al. 2011). In fact, this particular modification event, 6-*O* sulfation, is important for the function of hub HS (Levings et al. 2016). Compared to wild-type testes, the number of GSCs (Fig. 1.2b) and the rate of centrosome mispositioning (Fig. 1.2c) were both significantly higher in *HS 6-O sulfotransferases* (*Hs6st*) null mutants, mimicking *sfl* hub knockdown. Furthermore, overexpression of Sulf1, which removes 6-*O* sulfate groups, specifically in the hub showed the same defects, recapitulating the *sfl* and *Hs6st* knockdown phenotype.

How does hub HS non-autonomously affect the GSC asymmetric division? Analyses of hub-specific *sfl* knockdown and *Hs6st* mutant animals showed that hub HS is required for proper placement and function of the centrosome anchoring machinery (Levings et al. 2016). Therefore, perturbed HS function in the niche leads to a failure in the asymmetric division (centrosome positioning/spindle orientation). This causes a high frequency of symmetric division, which produces two stem cells.

1.2.5 HS in the Niche Prevents Tumor Formation in the Testis

The stem cell niche usually prevents abnormal behaviors of stem cells (Clarke and Fuller 2006; Hudson et al. 2013). Malfunction of niche signaling may predispose a transformation of stem cells into cancerous cells (Bhowmick et al. 2004; Radisky and Bissell 2004), including testicular germ cell tumors (Krausz and Looijenga 2008; Krentz et al. 2009; Gilbert et al. 2011).

A similar phenomenon has been found in *Drosophila* (Levings and Nakato 2018). Hub-specific knockdown of *sfl* causes not only the asymmetric division defect of GSCs described above, but also morphological defects at a lower penetrance. A fraction of hub-specific *sfl* knockdown testes exhibit a severe “tumorous” phenotype (Fig. 1.2d). In the wild-type testis, spermatogenic cells conventionally show the stereotypically ordered, progressive organization. In contrast, this ordered organization is disrupted in hub *sfl* knockdown testes. They often show the “ectopic stem cell” phenotype in which germline and somatic stem cells are abnormally located at distant positions from the hub. These observations indicate that the hub HS has the ability to retain stem cells locally near the niche.

How does hub HS affect the niche–GSC communications? The main pathway responsible for male GSC renewal is Jak/Stat signaling. *In vivo* analyses of Jak/Stat activity showed that hub-specific loss of HS resulted in abnormally higher levels of

Jak/Stat signaling levels in regions distant from the niche (Fig. 1.2e). This observation suggests that hub HS usually sequesters Upd, an HS-binding protein, at the niche. Therefore, a high level of Upd ligand is limited to hub-contacting GSCs. In the absence of hub HS, Upd becomes available in more distant regions, leading to germline tumors. Thus, HS functions as a sentinel to ensure the integrity of the niche organization and prevent tumorigenesis.

1.3 Heparan Sulfate Proteoglycans Regulate Stem Cell Replacement in the *Drosophila* Follicle Stem Cell Niche

1.3.1 The Drosophila *Ovarian Follicle Stem Cell Niche and Stem Cell Quality Control*

Stem cells are maintained throughout adult life, but they are not immortal cells. Individual stem cells have limited lifespans (Margolis and Spradling 1995). Adult stem cells in the niche are regularly replaced so that the niche is always occupied by young, healthy, and functional stem cells (Xie and Spradling 2000; Ryu et al. 2003; Nystul and Spradling 2007). This stem cell replacement can be achieved by competition for niche occupancy between stem cells and their direct progenitors (Nystul and Spradling 2007, 2010; Jin et al. 2008). This behavior of stem cell progenitors contributes to the stem cell quality control (Nystul and Spradling 2007, 2010).

Drosophila ovarian follicle stem cells (FSCs) have been used to study stem cell maintenance and competition (Sahai-Hernandez et al. 2012). All somatic follicle cells are produced by divisions of two FSCs, each of which is located in separate niches in the germarium (Margolis and Spradling 1995) (Fig. 1.1a). These FSCs are maintained through regular replacement: an FSC progenitor routinely migrates across the germarium and reaches a stem cell in the other niche (Nystul and Spradling 2007, 2010). There, these two cells compete for the niche occupancy, but the molecular basis for this competition is unknown. Through this replacement mechanism, the average half-life of wild-type FSCs is approximately 12 days. The phenomenon of stem cell competition was first observed in *Drosophila*, but it is now found in many stem cell types of different species (Li and Clevers 2010).

FSC competitive behavior can be examined in vivo using a lineage tracing technique (Lee and Luo 2001; Takemura and Nakato 2015). This FSC maintenance assay shows that GFP-labeled wild-type FSCs slowly disappear from the germarium due to normal turnover (Fig. 1.3a; Song and Xie 2003; Vied and Kalderon 2009; Vied et al. 2012; Zhang and Kalderon 2000; Kirilly et al. 2005). If the GFP-marked FSC is mutated in a gene essential for normal competition, the mutant progenitors will disappear more quickly than wild-type FSCs. If a mutation results in hyper-competition, on the other hand, the GFP-positive cells tend to occupy both niches. Once both niches are occupied with the hyper-competitive mutant cells, the GFP-positive progenitors expand into the entire epithelial sheet, which is called

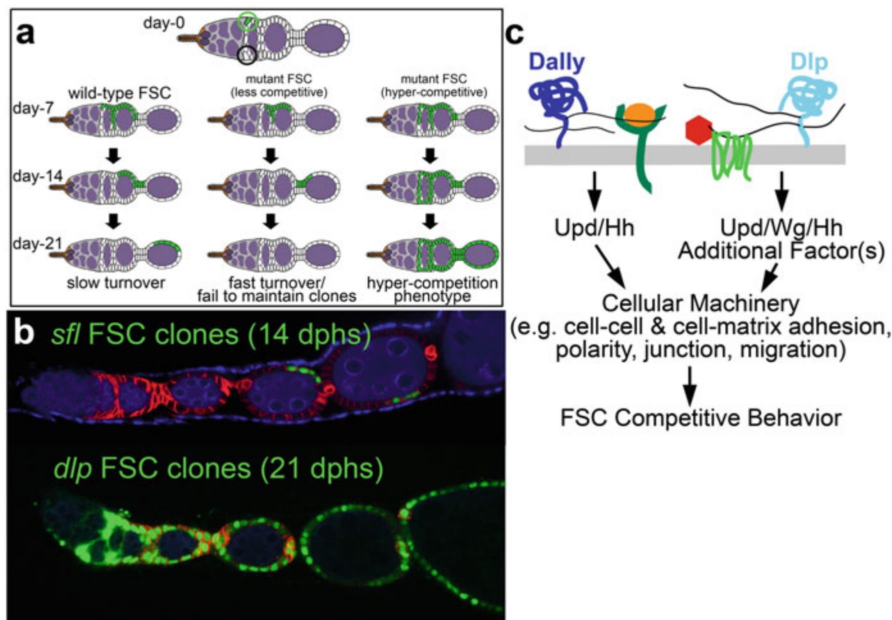

Fig. 1.3 HSPGs in the *Drosophila* follicle stem cell niche. (**a**) FSC lineage analysis. The GFP-positive mutant (or wild-type) FSC and its progenitors are shown in green. Unmarked cells represent wild-type cells. FSC is marked with GFP by heat shock-inducible DNA recombination. Once marked, its progenitors are permanently GFP-positive. Wild-type FSC and its progenitors slowly disappear due to normal turnover (left). If a GFP-labeled FSC is mutant for a gene essential for normal FSC competition, the progenitors will disappear more rapidly than wild-type (middle, less competitive phenotype). If a mutation in an FSC causes hyper-competition, on the contrary, both niches are likely to be occupied by mutant FSCs (right, hyper-competitive phenotype), leading to "all-marked" phenotype. (**b**) Ovarioles with *sfl* and *dlp* mutant FSC clones. (top) *sfl* FSC clones are quickly lost from the germaria. The ovariole bears only few *sfl* mutant progenitors (green) at 14 days after clone induction. *Dally* mutant FSCs show the same phenotype. (bottom) In contrast, *dlp* mutant cells show "all-marked" phenotype. These ovaries do not show any sign of abnormal growth. (**c**) Model for the role of glypicans in FSC competition. Dally and Dlp orchestrate multiple signaling pathways, including Upd, Wg, and Hh. Dlp may regulate additional factor(s). Together, these pathways affect downstream events such as cell adhesion and migration. The figures are modified from Su et al. (2018) with permission from the Genetics Society of America

the "all-marked" phenotype. This behavior of FSC progenitors is similar to the aggressive expansion of cells with pre-cancerous mutations during an early phase of cancer formation (Visvader 2011; Nystul and Spradling 2007; Shiozawa et al. 2011). This assay has been used to identify molecules and pathways that affect FSC replacement and competition (Sahai-Hernandez et al. 2012).

1.3.2 Glypicans Regulate Follicle Stem Cell Competition

In contrast to the non-autonomous functions of HSPGs in the niche cells for GSC control, HSPGs play autonomous roles in the FSCs. Two glypicans, *dally* and *dlp*, are expressed in the FSCs and control FSC competition for niche occupancy (Su et al. 2018). The lineage tracing assay demonstrated that *sfl* or *dally* mutant FSCs disappeared rapidly from the germaria (Fig. 1.3b), showing the necessity of Dally for normal FSC maintenance. Unexpectedly, *dlp* mutant FSCs behaved as a hyper-competitive mutant. *Dlp* mutant progenitors tend to occupy the niche, leading to the "all-marked" phenotype (Fig. 1.3b). Hence, the two *Drosophila* glypicans act in opposite ways during FSC competition and are involved in the FSC quality control. These results are interesting since different human glypican molecules also show opposite effects on cancer formation. Some glypicans are known to be oncogenic, like *dally*, whereas others behave as tumor suppressors, similar to *dlp* (Pilia et al. 1996; Cano-Gauci et al. 1999; Li et al. 2004; Williamson et al. 2007).

What is the molecular basis for the abnormal behaviors of glypican mutant FSCs? At this point, the answer is only partially obtained. FSC maintenance is known to be controlled by Jak/Stat, Hh, and Wingless (Wg: a *Drosophila* Wnt) signaling. The ligands of all these pathways are HS-dependent factors. Systematic analyses of *in vivo* signaling activities showed that *dally* RNAi knockdown in FSCs interfered with Jak/Stat and Hh signaling (Su et al. 2018). Similarly, the Jak/Stat, Hh, and Wg signaling pathways were impaired by *dlp* RNAi knockdown. The reduced Jak/Stat and Hh signaling is consistent with the compromised maintenance and competition of *dally* mutant FSCs (Vied et al. 2012). On the other hand, the mechanism of the hyper-competitive behavior of *dlp* mutant FSC remains to be determined. Interestingly, the overall morphology of *dlp* mutant ovaries is normal. This is a unique phenotype since known mutants that show the all-marked phenotype are typically accompanied by tissue overgrowth. This observation suggests that *dlp* might regulate an HS-dependent pathway that does not promote growth, such as Slit-Robo signaling. As shown in a model figure (Fig. 1.3c), the glypican co-receptors modulate the signaling output of HS-dependent factors, including Upd, Wg, and Hh, and yet unidentified molecules. Combined activities of these signaling pathways appear to consequently control downstream cellular events, such as cell adhesion and migration.

1.4 Heparan Sulfate Proteoglycans Regulate Stem Cell Activity in the *Drosophila* Midgut

1.4.1 The Drosophila *Midgut Intestinal Stem Cells*

Stem cell activity is tightly controlled during regeneration. Upon tissue damage, stem cell mitotic activity is substantially enhanced to replenish lost cells. When the

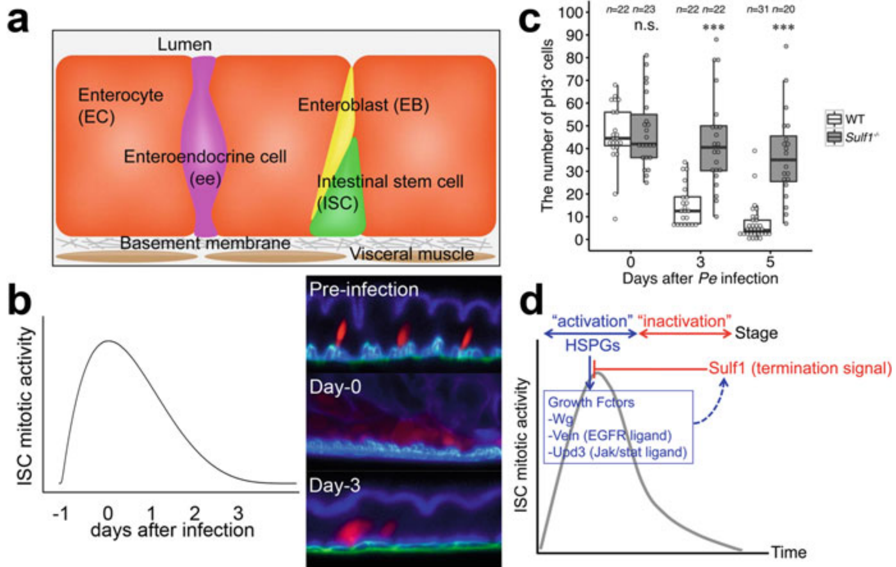

Fig. 1.4 HSPGs in the *Drosophila* midgut intestinal stem cells. (**a**) The *Drosophila* midgut. ISCs are localized at the basal side of the epithelium, contacting the basement membrane. (**b**) Time-course of midgut regeneration after *Pe* infection. (left) Number of mitotically active ISCs at different time points after infection (24 h feeding from −1 to 0). ISC mitotic activity peaks at 0–1 day and drops by day 3. (right) The midgut at pre-infection (top), day 0 (middle; right after the infection), and day 3 post-infection (bottom). *tdTomato* staining shows ISCs and progenitors (red). Cell cortex and the basement membrane are marked in blue (Phalloidin) and green (Trol-GFP), respectively. (**c**) The number of mitotically active cells at indicated time points after *Pe* infection. In *Sulf1* mutants, ISCs remain active for an extended period. (**d**) Model for the midgut regeneration termination. Upon tissue damage, HSPGs promote mitogenic signaling. At this stage, *Sulf1* expression is downregulated. *Sulf1* is re-expressed at the termination stage to reduce the levels of HS 6-*O* sulfation. This results in rapid shutdown of ISC division. Since *Sulf1* is induced by Wg signaling in the developing wing (Kleinschmit et al. 2010), it is possible that *Sulf1* expression at the termination stage is also induced by ISC activating pathways (blue dotted line). Figure 1.4c and d is modified from Takemura and Nakato (2017) with permission from the Company of Biologists

regeneration is completed, their proliferation rate comes back to the baseline. Insufficient stem cell activity results in impaired regeneration. On the other hand, the failure of proper inactivation of stem cell proliferation at the end of regeneration leads to tumor formation.

The *Drosophila* adult midgut has been used extensively to elucidate the molecular mechanisms of epithelial homeostasis and regeneration. The midgut contains two types of differentiated cells: the enterocytes and the secretory enteroendocrine cells (Micchelli and Perrimon 2006; Ohlstein and Spradling 2006). These cells are produced by intestinal stem cells (ISCs), which are found along the entire length of the midgut (Fig. 1.4a; Ohlstein and Spradling 2006; Micchelli and Perrimon 2006). The ISCs increase the rate of division in response to tissue damage (Fig. 1.4b; Amcheslavsky et al. 2009; Jiang et al. 2009). This regeneration can be readily

induced in the laboratory using a variety of chemical and bacterial insults. For example, enteric bacterial infection (*Pseudomonas entomophila, Pe*) activates several pathways, including Jak/Stat, EGFR, Wnt, and Hedgehog (Hh) signaling, to stimulate the repair (Jiang et al. 2016; Guo et al. 2016; Naszai et al. 2015).

Several lines of evidence indicated that HSPGs play a role in midgut homeostasis. The *Drosophila* perlecan, Trol, is required for the attachment of ISCs to the basement membrane and important for ISC maintenance during normal homeostasis (You et al. 2014). Furthermore, loss of an HS 3-*O* sulfotransferase results in the abnormal activation of ISC division *via* upregulated EGFR signaling (Guo et al. 2014). The role of HSPGs in regulating ISCs during homeostasis raised an interesting question: does HS contribute to controlling stem cell activity during regeneration?

1.4.2 HSPGs Regulate Damage-Induced Activation of ISCs

A study using the *Pe*-infection model demonstrated that *sfl* RNAi knockdown in the ISCs blocked the infection-induced increase of ISC division (Takemura and Nakato 2017). As a result, the RNAi animals failed to maintain the normal size and morphology of the midgut, leading to lethality. These observations demonstrated the critical requirement of HS function in ISC activation after bacterial infection. Additionally, silencing *Hs6st* by RNAi knockdown resulted in the same phenotype, showing that HS 6-*O* sulfation is essential for this process. These results are not surprising given that the ligands of mitogenic pathways mentioned above, which promote ISC division in response to tissue damage, are known HS-dependent factors. What is more interesting is a finding that a specific HS modification plays a key role in terminating ISC proliferative activity at the end of regeneration described below (Takemura and Nakato 2017).

1.4.3 Sulf1 Is Required for ISC Inactivation at Late Stages of Regeneration

Not only the activation of the proliferative capacity of stem cells but also its inactivation is equally crucial. The failure of proper termination of tissue regeneration results in the emergence of unwanted cells, leading to aberrant organ sizes (Miyaoka and Miyajima 2013) and an increased risk of cancer (Fuchs et al. 2013; Hsu and Fuchs 2012). Indeed, stem cell activity is precisely controlled at late phases of midgut regeneration. ISC proliferation rates are high for 2 days after *Pe* infection (activation stage) (Fig. 1.4b; Jiang et al. 2009). At this phase, newly emerged cells are stratified and the basement membrane becomes disorganized. The mitotic

activity rapidly decreases on day 3, when regeneration is completed (inactivation stage). By this time, the midgut reforms morphologically normal epithelium.

Quantification of ISC division throughout the course of regeneration revealed that *Sulf1* mutants fail to properly shut down ISC mitotic activity at the termination stage (Fig. 1.4c; Takemura and Nakato 2017). Thus, HS 6-*O* desulfation by *Sulf1* is required for the prompt termination of ISC proliferative capacity at the end of regeneration (Fig. 1.4d). *Sulf1* is secreted from enterocytes and visceral muscles surrounding the midgut epithelium. Interestingly, the expression level of *Sulf1* changes during regeneration; its expression is downregulated soon after regeneration is induced (activation stage) and returns to a normal level at the end of regeneration (inactivation stage). These findings suggested that *Sulf1* acts as a brake for ISC mitotic activity during normal homeostasis and at the regeneration termination. By its activity to remove the ligand-binding sites on HS, Sulf1 contributes to rapid downregulation of mitogen signaling, ensuring appropriate termination.

Despite the significance of the inactivation of stem cell proliferative capacity at the end of regeneration, the molecular basis for this process is poorly understood. One apparent termination mechanism of ISC division is the transcriptional inactivation of the mitogenic signals (Upd3, Vein, and Wg). Sulf1's ability to rapidly shut down multiple ISC mitogen pathways provides an additional control system to halt stem cell division. This finding indicates that HS 6-*O* desulfation plays important roles not only in stem cell control but also in cancer formation. The function of *Drosophila Sulf1* in the midgut is consistent with the involvement of mammalian Sulfs in ovarian, breast, lung, pancreatic, and hepatocellular cancers (Khurana et al. 2013; Lemjabbar-Alaoui et al. 2010; Nawroth et al. 2007; Lai et al. 2003, 2008).

1.5 Concluding Remarks

When two cells communicate, it is obvious that a signaling ligand and its receptor are both required. However, why do such signaling modules typically possess a third component, “co-receptors”? *In vivo* studies of HSPGs, a major class of co-receptors conserved throughout all animal species, using the *Drosophila* model have provided new insights into this classical question. First, during animal development, the signaling outcome is not just ON or OFF, but individual cells must implement a quantitative and precisely controlled signaling dosage. HSPG co-receptors are required for this adjustment: they act as a “rheostat” for fine control of signaling, converting a signaling pathway from a “switch system” to a “dial system.” This is reflected by the fact that virtually all known morphogen molecules, which function in a concentration-dependent manner, are HS-dependent factors. Second, HSPGs contribute to positive and negative feedback loops of these pathways, providing the robustness of morphogen signaling, and thus morphogenesis. Again, the robustness is a unique and important characteristic of morphogen systems, although its molecular mechanisms are poorly understood. Finally, HSPGs regulate the spatial distribution of signaling ligands in a tissue as well as the signal reception on the cell

surface. For example, HSPGs concentrate such ligands in the stem cell niche, a small area of a tissue, to help create a special microenvironment for stem cell maintenance. *Drosophila* genetics has revealed that HS-mediated cell communications are indeed effectively used in morphogen signaling and the stem cell niche. Since fundamental principles of HS structure and function are conserved from fruit flies to human, proteoglycan studies using *Drosophila* genetics will provide novel insights into therapeutics and diagnostics for HS-related diseases, such as cancers.

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Chapter 2
Proteoglycans in Zebrafish Development

Judith Habicher, Beata Filipek-Górniok, Lena Kjellén, and Johan Ledin

Abstract Forward genetic screens have identified several zebrafish mutants related to glycosaminoglycan biosynthesis with developmental defects. Many important studies have also used morpholino knockdown of gene expression for functional studies, but off-target effects may be difficult to predict. However, the introduction of the CRISPR-Cas9 genome editing method has now made it possible to generate genetic knockouts overcoming this problem. We know that more heparan sulfate (HS) than chondroitin sulfate (CS) is produced during early zebrafish development while at later stages the dominance of CS is massive. Since a majority of the identified mutants affect production of both HS and CS, their respective roles are sometimes difficult to discern. The two glycosaminoglycans (GAGs) may also have overlapping and redundant functions, such as supporting growth factor signaling, adding to the picture. Craniofacial phenotypes are present in many of the mutants with defective organization and morphogenesis of chondrocytes and bone. Other phenotypes include impaired axon sorting, disturbed heart development, and absent or partly developed pectoral fins. Morpholino studies have also indicated specific functions in angiogenesis, vasculogenesis, and left-right patterning which await confirmation using genetic knockouts. The chapter focuses on HS and CS, but some results also concern hyaluronan and keratan sulfate.

All cells in every organism originate from totipotent stem cells of the zygote. The coordinated stem cell maturation process ensures correct assembly of tissues and organs during organism development. The regulation of this process is highly influenced by the microenvironment of each stem cell, commonly referred to as

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the stem cell niche. In the niche, the often-unique combinations of extracellular mechanisms will cause transcriptional and epigenetic regulation changing behavior of the stem cells. Major extracellular mechanisms include cell–cell and cell–matrix contacts as well as signaling through juxtacrine and paracrine mechanisms. Proteoglycans at the cell surface and in the extracellular matrix are important components of the niche.

2.1 Studying Development in Zebrafish

Zebrafish offer unique features to study cell behavior during vertebrate development. The model system is inexpensive, a mating produces large numbers of fertilized eggs, and the zebrafish larva is transparent (Fig. 2.1) and small enough to allow time-lapse imaging at single cell resolution.

Thousands of zebrafish transgenic lines which express detectable markers in specific cell populations have also been developed by the zebrafish community (Fig. 2.2). The transgenic larva can be dissolved and fluorescent cells sorted by FACS methodology and subjected to transcriptomic and proteomic analysis.

Fig. 2.1 Stages of zebrafish development. Zebrafish embryos develop in a transparent egg shell and the cells on top of the yolk divide rapidly within the first hours post-fertilization (hpf). At 6 days post-fertilization (dpf) the larvae are already swimming and feeding, but it takes approximately 2–3 months for the fish to reach sexual maturity

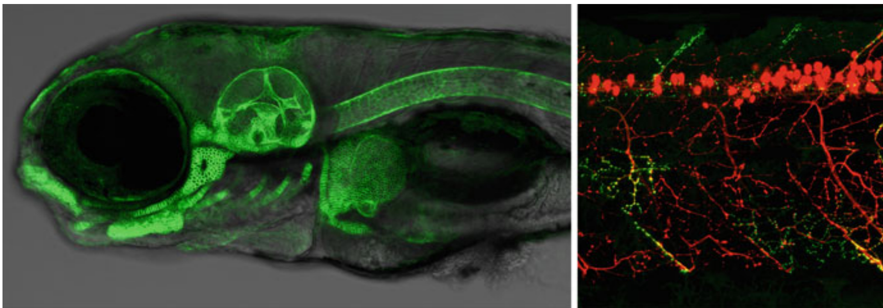

Fig. 2.2 Transgenic zebrafish lines. Genetically modified zebrafish with fluorescent proteins in combination with different microscopy techniques allow for the study of specific cells within the developing organism in vivo. Image to the left: A lateral view of zebrafish head at 5 dpf shows a membrane localized GFP (green fluorescent protein) expressed under the col2a1a promoter visualizing cartilage cells (Dale and Topczewski 2011). Image to the right: The spinal cord of a 3-day-old larva where motorneurons are labelled red with td:Tomato fluorescent protein (Seredick et al. 2012) while the synaptic terminals are labelled green with synaptophysin (Meyer and Smith 2006). (Image is a courtesy of Harmen K. Koning)

Recently developed tools such as genetic linage tracing and single cell transcriptomics have given information of in situ stem cell development, unimaginable a few years ago (McKenna et al. 2016; Farrell et al. 2018; Wagner et al. 2018).

2.2 Loss of Gene Function Experiments in Zebrafish

The introduction of random mutations in the zebrafish has for a long time been used for forward genetic screens where mutagens randomly introduced loss-of-function mutations in the zebrafish genome. A total of nine genetic mutants with defective GAG biosynthesis have been identified. With the exception of two HS glycosyltransferases (*ext2* and *extl3*) mutants, the other mutations are present in genes encoding proteins important for precursor availability (*ugdh, papst1, uxs1*) or synthesis of the shared HS and CS linkage region (*fam20b, xylt1, b4galt7, b3gat3*). From the early 2000s, the primary reverse genetic tool in the zebrafish model system was morpholinos, oligomer molecules with DNA bases attached to a synthetic backbone which can be designed to block translation and splicing of specific genes. Over the years, morpholinos have been used in thousands of publications from the zebrafish field due to their affordability and ease to use. However, morpholino knockdown experiments must be executed with appropriate controls to ensure specificity and avoid unspecific effects. In the late 2010s, targeted mutation using CRISPR/Cas9 technology became a widespread method for loss of gene function experiments. Striking discrepancies between phenotypes observed after morpholino injections and genetic knockouts (Kok et al. 2015) suggested that the contribution of unspecific morpholino effects might have been far more extensive than previously anticipated. However, in seminal studies by the Stanier lab a previously unknown mechanism named genetic compensation was presented which could explain the discrepancies (Rossi et al. 2015; El-Brolosy et al. 2019). Genetic compensation would occur when an mRNA containing a generated premature stop codon was committed to nonsense mediated decay. As a result, genes with a similar sequence would be selectively upregulated. In this manner, a knockdown using morpholino injections where no genetic compensation would occur could result in a more severe phenotype than a genetic knockout. At present, however, it is not known how common the genetic compensation mechanism is. It can be anticipated that our understanding of differences between knockout and knockdown experiments will continue to evolve in the coming years.

In this chapter, we will concentrate on data obtained using mutants but also mention results obtained using morpholino knockdown approaches (the reader is advised to consider recent recommendation for the use of morpholinos in zebrafish (Stainier et al. 2017)).

2.3 Glycosaminoglycan Biosynthesis

Except for hyaluronan, all glycosaminoglycans (GAGs) are polymerized and modified in the Golgi compartment of cells, linked to proteoglycan core proteins (Lindahl et al. 2017). Heparan sulfate (HS), heparin, chondroitin sulfate (CS), and dermatan sulfate (DS) all contain the same O-linked tetrasaccharide, Xyl-Gal-Gal-GlcUA, connecting a serine in the core protein to the GAG chain. The linkage tetrasaccharide can also be modified by phosphorylation and sulfation (Lindahl et al. 2017). In contrast, keratan sulfate (KS) uses three different ways to attach to proteins (Caterson and Melrose 2018). In the cornea, KS is N-linked to its core proteins, while cartilage KS is O-linked through an N-acetylgalactosamine and brain KS is attached via an O-linked mannose. The biosynthesis of KS will not be covered in this chapter (see Caterson and Melrose 2018 and references therein).

Heparin and HS have the same basic structure where glucosamine residues (*N*-sulfated or *N*-acetylated) alternate with hexuronic acid (glucuronic acid, GlcA, or its C5-epimer iduronic acid, IdoA). The two GAGs also contain O-sulfate groups in different positions (see Fig. 2.3). HS, present on the surface of most cells in the vertebrate body, has a lower average content of sulfate groups than heparin (HS, $\approx$1 sulfate group/disaccharide; heparin, 2–2.5 sulfate groups/disaccharide, Gallagher and Walker 1985). While heparin in mammals seems to be confined to mast cells, it is not known if heparin is produced by zebrafish. CS and DS are also closely related. They both contain *N*-acetylgalactosamine which in CS alternate with glucuronic acid, while DS contains both glucuronic acid and iduronic acid (Lindahl et al. 2017). In many types of structural analyses, glucuronic acid cannot be distinguished from iduronic acid. Therefore, CS is often used as a collective term to describe both CS and DS. The average sulfate content of CS and DS is often similar to that of HS.

While a majority of proteins are glycoproteins, i.e., contain covalently bound monosaccharides, oligosaccharides, or more extended saccharides, only a few proteins in mammals, $<$100, carry GAG side chains. Most core protein attachment sites are specific for a certain GAG, while a few are promiscuous and can alternate between different GAGs, such as HS and CS/DS. The whole-genome duplication that took place in the common ancestor of all extant teleosts including zebrafish has resulted in duplicate genes compared to e.g. mammals (Meyer and Van de Peer 2005). While not all duplicated genes have been retained, some proteoglycan-related genes are found in duplicates (indicated with blue bars in Fig. 2.3).

During HS and heparin biosynthesis, alternating *N*-acetylglucosamine and glucuronic acid residues will be added by the Ext1/Ext2 copolymerase after addition of the first *N*-acetylglucosamine residue to the linkage tetrasaccharide by another glycosyltransferase, Extl3 (Kreuger and Kjellen 2012). This is a critical step which will enable HS biosynthesis and prevent CS/DS from being synthesized. At the same time as the HS chain is growing, it is modified by sulfotransferases and an epimerase

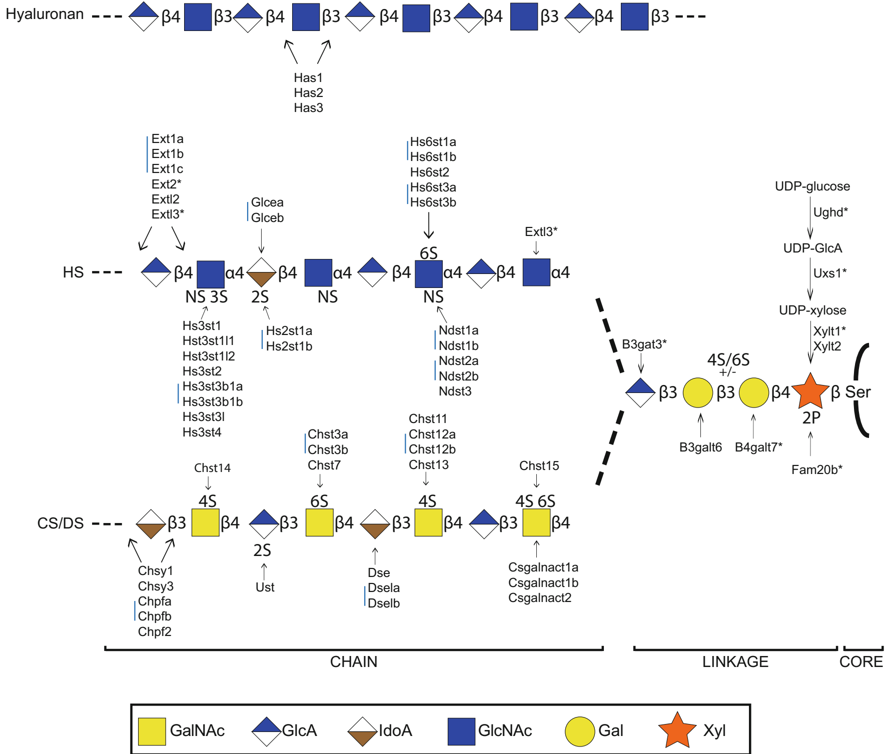

Fig. 2.3 GAG biosynthesis in zebrafish. *Hyaluronan*: Synthesis takes place at the plasma membrane where hyaluronan synthases transfer *N*-acetylglucosamine (GlcNAc) and glucuronic acid (GlcA) in alternating sequence to the growing chain. *HS and CS/DS linkage region:* A xylosyltransferase transfers the first sugar to a serine residue in the proteoglycan core protein. Two different galactosyltransferases then add one galactose residue each followed by the addition of a GlcA. The two galactose residues may be sulfated and the xylose residue phosphorylated. Shown are also the two enzymatic steps needed to produce UDP-xylose, the xylosyltransferase substrate. *HS elongation and modification*: A GlcNAc-transferase, Extl3, initiates HS biosynthesis while HS-copolymerases composed of an Ext1 and an Ext2 subunit build the GAG chain by addition of alternating units of GlcNAc and GlcA. Bifunctional *N*-deacetylase/*N*-sulfotransferases initiate the modification process by replacing the GlcNAc acetyl group with a sulfate group followed by C5-epimerization of GlcA into iduronic acid (IdoA). *O*-Sulfation at different positions is then carried out by HS 2-*O*-sulfotransferases, HS 6-*O*-sulfotransferases, and HS 3-*O*-sulfotransferases. *CS/DS elongation and modification*: Transfer of an *N*-acetylgalactosamine (GalNAc) to the linkage region by a GalNAc-transferase starts CS/*DS* biosynthesis, where complexes of chondroitin synthases and chondroitin polymerization factors build the CS/*DS* chain. A single CS/*DS* 2-*O*-sulfotransferase and several CS 4-*O*- and 6-*O*-sulfotransferases add sulfate groups to the growing chain while C5-epimerases form IdoA from GlcA, thereby converting CS to DS. Blue bars indicate enzymes encoded by two zebrafish genes orthologous to a single mammalian gene. A star indicates that a genetic mutant for this enzyme is available. *Xyl* xylose, *Gal* galactose, *GlcNAc* N-acetylglucosamine, *IdoA* iduronic acid, *GlcA* glucuronic acid, *GalNAc* N-acetylgalactosamine

(Fig. 2.3). CS/DS synthesis occurs in a similar manner. After the step committing the biosynthesis to CS/DS, addition of an *N*-acetylgalactosamine to the linkage region, transfer of alternating glucuronic acid and *N*-acetylgalactosamine residues is performed by dimeric combinations of six different polymerases. Sulfotransferases and, in the case of DS biosynthesis, two epimerases modify the growing galactosaminoglycan chain. The biosynthesis of KS will not be covered in this chapter (see Caterson and Melrose for a recent review).

2.4 Structure and Amounts of HS and CS/DS During Zebrafish Development

While HS dominates during the first hour after fertilization, equal amounts of CS/DS and HS are found 24 h post-fertilization (Fig. 2.4; (Filipek-Gorniok et al. 2013)). The amounts of CS/DS continue to increase and dominate during later phases of development. While the overall structure of HS is fairly constant over the period (measured in whole embryos/fish), CS 4-*O*-sulfation dominates during the first two days of development, after which 6-*O*-sulfation takes over (Habicher et al. 2015).

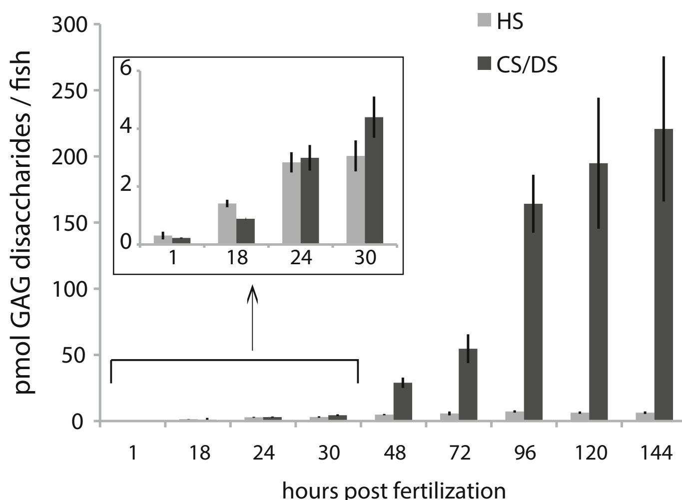

Fig. 2.4 HS and CS/DS in zebrafish development. From 1 to 30 h post-fertilization both HS and CS are steadily increasing, after which CS/DS continues to increase drastically while HS reaches a steady state (modified from Filipek-Gorniok et al. 2013, with the permission of Wiley)

2.5 Maternal Contribution

As mentioned above, a total of nine genetic mutants with defective GAG biosynthesis have been identified in forward genetic screens. Mutations in seven of these genes will affect synthesis of both HS and CS/DS resulting in complex phenotypes of the mutants (see Fig. 2.3). Adding to the complexity is the fact that transcripts of genes important in early zebrafish development are often maternally contributed and will only over time be replaced by zygotic transcription. In studies of homozygous mutants from crossings between heterozygous individuals, equal amounts of wild-type and mutant transcript will be available for the embryo which will not lack functional protein until later in development. How long the effects of maternally contributed transcript will last varies, but maternal mRNAs are typically degraded within six hours while functional proteins as well as newly produced GAGs might be around for longer periods of time.

2.6 Craniofacial Phenotypes

A recurring phenotype when GAG biosynthesis is reduced in zebrafish embryos is deformed branchial arches and abnormal pharyngeal cartilage and jaw morphology, but additional distinct phenotypes have also been reported. Individuals with the *ugdh*$^{-/-}$ genotype, with reduced levels of UDP-glucuronic acid used for synthesis of both HS, CS/DS, and hyaluronan (see Fig. 2.3), develop branchial arch and jaw defects (Fig. 2.5a) and are unable to initiate cardiac valve formation (Walsh and Stainier 2001). In maternal-zygotic *ugdh*$^{-/-}$ individuals, lacking maternal contribution of the *ugdh* transcript, Fgf, Shh, and Wnt signaling is affected resulting in broad effects on patterning and morphogenesis (Superina et al. 2014).

Most functions of GAGs depend on interactions with proteins recognizing sulfation patterns on the GAG chain (Kjellen and Lindahl 2018). In *papst* mutants, where transport of the sulfate donor PAPS into the Golgi stacks is hampered, and where HS, CS/DS, and KS sulfate content is reduced, chondrocytes in the pharyngeal cartilage fail to intercalate (Clément et al. 2008). Also, *uxs1* mutants with defective production of UDP-xylose develop strong craniofacial phenotypes with defective organization and morphogenesis of chondrocytes and bone (Eames et al. 2010; Fig. 2.5b). Mutants in *xylt1* and *fam20*, responsible for addition and phosphorylation, respectively, of the xylose residues that link HS and CS/DS chains to the serine residues of the core proteins, develop abnormal skeletons and show delayed chondrocyte maturation and endochondral ossification (Eames et al. 2011). The *b4galt7* and *b3gat3* mutants, lacking the enzymes adding the first and second galactose residues to the HS and CS/DS linkage region, also develop abnormal jaw and pharyngeal cartilage structures (Amsterdam et al. 2004; Wiweger et al. 2011; Holmborn et al. 2012), making this phenotype strikingly consistent in all identified mutants affecting both HS and CS/DS biosynthesis. The observed

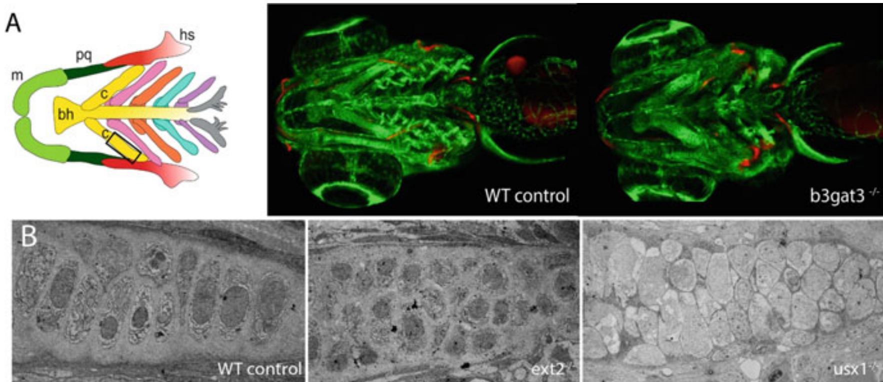

Fig. 2.5 Pharyngeal cartilage elements and chondrocyte morphology. (**a**) Schematic overview relating to the ventral views of the head of 6-day-old zebrafish larvae. Tg(fli1:GFP) and alizarin red stained larvae show the malformations, in particular the failed stacking of chondrocytes prominent in the ceratohyal of *b3gat3* mutants compared to wild-type fish. bh: basihyal, c: ceratohyal, hs: hyosymplectic, m: Meckel's cartilage, pq: palatoquadrate. (**b**) Zebrafish larvae at 4 dpf where both *ext2* and *uxs1* mutants show failed stacking of the chondrocytes within the ceratohyal compared to wild-type control, as shown in these transmission electron microscopy images of the area within the rectangle in A

phenotype of these mutants varies in severity, probably reflecting on how long the effects of maternal contribution of the respective proteins last (discussed above). Notably, while the structure of both HS and CS/DS is altered in *uxs1* and *b3gat3* mutants, the amount of HS produced is reduced to half, while only a small fraction of CS/DS is found in mutants compared to control larvae (Holmborn et al. 2012). While the phenotypes of mutants in enzymes acting early in GAG biosynthesis clearly illustrate the importance of GAGs for normal animal development, studies on genes encoding proteoglycan core proteins and enzymes acting later in either HS or CS/DS biosynthesis or degradation will be necessary to better understand the specific roles of different proteoglycans and GAG structure in zebrafish development.

2.7 Phenotypes Associated with Altered HS Production

Zebrafish phenotypes generally associated with altered HS levels or synthesis observed both in genetic mutants and in morpholino knockdowns include disturbed development of craniofacial cartilage, lack of (or shortened) pectoral fins, disturbed axon sorting, aberrant blood circulation, and vascular development. Mutations, genetic knockouts, or morpholino knockdowns of *ext2* are predicted to affect the amount of HS produced since Ext2 together with Ext1 form the HS-copolymerase responsible for elongation of the HS polysaccharide chain during biosynthesis (see above). The *ext2* gene has a widespread expression during development similar to that of *extl3,* encoding the glycosyltransferase which adds the first GlcNAc to the

linkage region (Fig. 2.3; Lee et al. 2004). In addition, maternal mRNA secures HS synthesis in the early embryo, most likely explaining why zebrafish *ext2* mutants survive and go through gastrulation while mouse embryos lacking Ext2 fail to gastrulate and die early during development (Stickens et al. 2005). However, as shown in Holmborn et al. 2012, in addition to reduced levels of HS in *ext2* and *extl3* mutants, the HS produced is supersulfated and increased levels of CS are found in the *extl3* but not in the *ext2* mutants. These results highlight the complex regulation of GAG biosynthesis and the difficulties in translating deficiency of a certain enzyme to GAG composition. Is the altered phenotype in *extl3* mutants due to less HS, increased sulfation of HS, or increased amounts of CS?

As discussed above, development of neural-crest derived cells to chondrocytes, forming the pharyngeal arches and the jaw is particularly dependent on expression of genes involved in GAG biosynthesis (Fig. 2.5b). In addition to the enzymes involved in synthesizing UDP-sugars, building the GAG-linkage region, or the protein transporting the sulfate donor PAPS into the Golgi compartment, the HS biosynthesis enzymes Ext2 and Extl3 have been shown to be necessary for craniofacial cartilage formation (Clément et al. 2008; Holmborn et al. 2012). A morpholino knockdown study of the HS biosynthesis enzyme Ndst1b function confirmed HS involvement in the process (Filipek-Górniok et al. 2015). In addition, tooth development is affected in *ext2* mutants (Wiweger et al. 2012). Several of the GAG biosynthesis mutants have impaired pectoral fin development, but the strength of the phenotype varies (Holmborn et al. 2012), probably due to different extent of maternal contribution. Pectoral fins are absent in *ext2* mutants due to impaired Fgf10 signaling, while Fgf4 and Shh signaling is functional, indicating a specificity in HS regulation of growth factors with HS affinity (Norton et al. 2005; Fischer et al. 2011). Early studies of *ext2* and *extl3* mutants demonstrated that their expression was required for axon sorting in the optic tract (Lee et al. 2004). Later studies demonstrated that during optical tract development sorting is achieved through degeneration of missorted axons and that expression of *ext2* is necessary for this process (Poulain and Chien 2013).

Only one genetic mutant in a proteoglycan core protein has yet been described in zebrafish, the glypican Gpc4, a cell surface proteoglycan modified with HS. In *gpc4*$^{-/-}$ embryos, the control of normal cell polarity during convergent extension movements is lost, probably due to reduced Wnt11 signaling (Topczewski et al. 2001). Embryos with the *gpc4*$^{-/-}$ genotype also show migrating lateral line primordium defects due to misregulation of a Wnt/β-catenin/Fgf signaling feedback loop (Venero Galanternik et al. 2016), similar to what is observed in *ext2*/*extl3* double mutants (Venero Galanternik et al. 2015). Cartilage cells also fail to intercalate and extend cartilage elements—a phenotype reminiscent of *ext2* mutants suggesting that the HS portion of Gpc4 is necessary for pharyngeal cartilage development (Wiweger et al. 2011). Subsequent studies have shown how Gpc4 regulates migration of endoderm cells (Hu et al. 2018), hair cell orientation (Navajas Acedo et al. 2019), cardiomyocyte differentiation (Strate et al. 2015), and cartilage and palate morphogenesis (LeClair et al. 2009; Rochard et al. 2016). This illustrates the multitude of

functions affected by the removal of Gpc4, often caused by impaired Wnt and Fgf signaling.

2.8 Morpholino Studies

Left-right patterning in early zebrafish development is controlled by Kupffer's vesicle where each cell develops a special apical cilium which moves fluid asymmetrically enabling the patterning. An elegant morpholino knockdown study implicated the HS proteoglycan syndecan-2 (Sdc2) in the process (Arrington et al. 2013), supporting earlier studies in Xenopus (Kramer and Yost 2002). Also, 3-*O*-sulfation of HS was shown to regulate left-right patterning (Neugebauer et al. 2013). In another morpholino study, knockdown of Sdc2 resulted in defect vasculogenesis (Chen et al. 2004). The importance of HS for zebrafish angiogenesis was also demonstrated by morpholino knockdown of HS 6-*O*-sulfotransferases (Chen et al. 2005) and by using a sugar analogue interfering with HS biosynthesis (van Wijk et al. 2013). It will be important to confirm these results with genetic knockouts of the genes encoding the core protein and biosynthesis enzymes.

2.9 Genetic Mutants in CS/DS Biosynthesis Remain to Be Described

While forward genetic screens have identified a number of enzymes involved in the biosynthesis of the linkage region and HS as important for zebrafish development, no genetic knockout of CS/DS biosynthesis enzymes or core proteins has yet been reported. However, morpholino knockdown of the CS polymerase Chsy1 affects Bmp and Notch signaling leading to impaired skeletal, pectoral fin, and retinal development (Li et al. 2010; Tian et al. 2010). Other morpholino experiments have described the requirement of *chsy1* for normal patterning of the cardiac atrioventricular boundary (Peal et al. 2009), a phenotype distinct from the cardiac phenotype described in the *uxs1* genetic knockout mutants (Walsh and Stainier 2001) discussed above. Morpholino knockdown of Chst11, a CS/DS sulfotransferase, has also been shown to affect motor axon guidance and development of somatic muscles (Wang et al. 2008).

2.10 Hyaluronan

This non-sulfated glycosaminoglycan is synthesized at the plasma membrane by hyaluronan synthases encoded in mammals by three genes (see Fig. 2.3). The zebrafish genome also contains three hyaluronan synthases, *has1*, *has2*, and *has3* (Bakkers et al. 2004). Of the three, only *has2* is expressed during embryogenesis, while has1 and has3 expression is initiated in 2-day-old larvae (Bakkers et al. 2004). As demonstrated by these authors, the expression pattern of *has2* matches the distribution of hyaluronan in the 10-somite stage embryo, supporting the notion that this enzyme is responsible for the first production of hyaluronan in the embryo. Morpholino knockdown of *has2* indicated that the lack of hyaluronan resulted in impaired migration during gastrulation and somite morphogenesis and also affected primordial germ cell migration (Bakkers et al. 2004). In addition, recent results demonstrate that development of the heart and in particular the atrioventricular valves depend on the controlled degradation of hyaluronan by a transmembrane protein with hyaluronidase activity, *tmem2* (Hernandez et al. 2019).

2.11 Concluding Remarks

Proteoglycans are likely to influence paracrine and juxtacrine signaling in all zebrafish stem cell niches, and, as discussed in this chapter, mutants where glycosaminoglycan synthesis is disturbed show severe developmental defects. Yet, little is so far known about the roles of specific proteoglycans/GAGs in zebrafish stem cell proliferation and differentiation. However, we anticipate that our understanding of the role of proteoglycans in zebrafish stem cell biology will develop rapidly and significantly in the coming years due to innovative method developments in single cell transcriptomics, CRISPR-Cas9 genome modification, and high-throughput phenotypic analysis.

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Chapter 3
The Pivotal Role of Versican Turnover by ADAMTS Proteases in Mammalian Reproduction and Development

Suneel S. Apte

Abstract The chondroitin sulfate proteoglycan versican is an important component of the provisional extracellular matrix present in the early mammalian embryo and ovarian follicle. This matrix is highly dynamic and is remodeled by several members of a class of secreted metalloproteinases, the ADAMTS proteinases. Accordingly, analysis of mouse mutants of ADAMTS proteases capable of versican processing has demonstrated a strong association of versican accumulation with the specific mutant phenotypes. These phenotypes include female infertility resulting from anovulation, developmental defects, e.g., open neural tube, cleft palate, cardiac anomalies, soft tissue syndactyly, and umbilical cord anomalies, as well as impaired parturition. Thus, ADAMTS proteolysis of versican has crucial roles in mammalian reproduction and morphogenesis.

3.1 The Provisional Extracellular Matrix of the Mammalian Embryo

Following sperm–egg fusion, the resulting mammalian embryo rapidly undergoes countless cell divisions to generate discrete cell “collectives.” Cells are organized within these collectives not only by cell–cell adhesion, but also by an extracellular matrix (ECM), which has multiple roles. It partitions the germ layers in the early embryo and subsequently organizes the various lineages and differentiated cell types in developing organs, provides structural support and an adhesive surface, ensures correct cell polarity, transmits cellular force, and stores and releases growth factors. The earliest cell collectives are sheets. They subsequently undergo complex morphogenetic movements such as tube formation and branching morphogenesis of the tubes, which would not be possible without their ECM. Normal embryogenesis requires completion of profoundly complex cell migration events, e.g., neural crest

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cell migration and germ cell migration. The ECM is also thought to have an important role in cell guidance during migration by providing adhesive cues, and equally importantly, anti-adhesive cues (Dutt et al. 2006).

Ontogenetically, the earliest organized ECMs of embryos are basement membranes, which endow cells with the ability to form sheets and tubes, echoing evolution of the first multicellular eukaryotes. As the embryo grows, most of its non-epithelial and non-neural cells, i.e., mesenchymal cells, are surrounded by abundant interstitial ECM. Interstitial ECM, especially in the early embryo, is highly hydrated and is not dominated by the major ECM components that characterize adult tissues, such as collagen fibrils, and does not contain elastic fibers. Instead, embryonic ECM is relatively rich in versican (Zimmermann and Ruoslahti 1989), a chondroitin sulfate proteoglycan also known as PG-M (Kimata et al. 1986) that is a member of the large aggregating proteoglycan family and like the other members binds to the glycosaminoglycan hyaluronan (HA) (LeBaron et al. 1992). Aggregates of HA and versican have a net negative charge that draws in sodium counter-ions accompanied by water, conferring a swelling property to the ECM (referred to as the Donnan equilibrium) (Roccabianca et al. 2014). The HA-versican aggregates are linked to fibronectin, another abundant early ECM component, via the versican G3 domain (Wu et al. 2005). Fibronectin is thought to be a key foundational molecule in the ECM (Singh et al. 2010), where it guides and facilitates deposition of collagen fibrils, basement membranes, and fibrillin microfibrils, which in turn provide a scaffold for elastic fiber formation. The highly hydrated ECM is also permissive for cell migration, since it provides less of a physical barrier than a collagen-rich ECM. However, this ECM is not mechanically suited for the demands of postnatal life and is gradually replaced from late development onward. Thus, the embryonic interstitial ECM is a temporary or provisional ECM that is crucial for normal development. A provisional ECM may persist as the pericellular matrix of many mesenchymal cell types in the adult, and reappears during processes that involve rapid remodeling, repair, and regeneration (Wight 2017).

The importance of each of these major provisional ECM molecules for morphogenesis is clear from in vivo studies showing that targeted inactivation of *Vcan*, *Has2* (the primary hyaluronan synthase in the embryo), and *Fn1* (encoding fibronectin) each lead to early lethality in the mouse embryo (around gestational age (E) 9.5 days to E10.5) (Camenisch et al. 2000; George et al. 1993; Mjaatvedt et al. 1998). *Has2* and *Vcan* mouse mutants have similar hearts defects, consistent with them working as an obligate complex, whereas $Fn1^{-/-}$ embryos have abnormal vasculature (Camenisch et al. 2000; George et al. 1993; Mjaatvedt et al. 1998). One may speculate that along with evolution of the embryonic provisional matrix to support morphogenesis, specific proteolytic and cellular mechanisms must have co-evolved to disassemble it and that such activities would appear during replacement of provisional by adult ECM. Disassembly may occur *en masse* and in an accelerated timescale during tissue sculpting processes, e.g., cardiac valve sculpting or interdigital web regression, which lead to mature valve leaflets and separated digits respectively. Alternatively, it may be subtle and occur over longer timescales, and as such, be barely noticeable. In tadpoles, a transient free-swimming amphibian

developmental stage, mechanically strong collagen-rich matrices are already well developed in the tail and fins, which are removed during metamorphosis. Regression of these vestigial structures, a defining landmark in amphibian metamorphosis, occurs via their rapid, *en masse* removal with a major requirement being local expression of a collagenase (Gross and Lapiere 1962). The demonstration and subsequent characterization of this collagenase is of immense historical significance for the metalloproteinase field and provided a precedent for the possibility that specific proteolytic activities were similarly necessary for digesting the collagen-poor provisional matrix during mammalian embryogenesis. This has turned out to be correct, and versican turnover by a specific protease class, the *A disintegrin-like and metalloprotease domain with thrombospondin type 1 repeats* (ADAMTS) proteases (Apte 2009), is now accepted to have an important role in provisional ECM remodeling (Dubail and Apte 2015; Nandadasa et al. 2014). In this chapter, I will discuss the biological impact of versican turnover by ADAMTS proteases in mammalian reproduction and development, providing several examples that illustrate the necessity and significance of this process. Additional details on the role of versican and ADAMTS proteases in embryos were provided in a review on versican and ADAMTS proteases written a few years ago (Nandadasa et al. 2014).

3.2 Overview of the ADAMTS Proteases and Versican

All ADAMTS proteases are secreted enzymes. Their catalytic domain is of the snake venom (reprolysin) type, so in this respect they are more closely related to ADAM (A disintegrin and metalloprotease domain) proteases than matrix metalloproteinases (MMPs). However, all ADAMs are membrane-anchored and are principally involved in ectodomain shedding, whereas MMPs and ADAMTS proteases have a major role in ECM proteolysis. Moreover, in contrast to ADAMs, the ADAMTS protease domain structure includes one or more thrombospondin type 1 repeats.

Phylogenetic analysis has shown that the ADAMTS family contains seven homologous protease pairs and one set of three homologous proteases, indicative of evolution by duplication of ancestral genes (Huxley-Jones et al. 2005). Paired ADAMTS proteases have identical domain structures and a high primary sequence similarity. The information that has emerged to date suggests that enzymes in a pair show overlapping expression and cooperative functions, and limited evidence suggests that such closely related proteases may have overlapping substrate preferences. Among them, ADAMTS1, ADAMTS4, ADAMTS5, ADAMTS8, ADAMTS9, ADAMTS15, and ADAMTS20 are known to cleave proteoglycans (Dancevic et al. 2013; Longpre et al. 2009; Sandy et al. 2001; Somerville et al. 2003). Each, other than ADAMTS8, has been shown to cleave versican, but since ADAMTS8 cleaves aggrecan (Collins-Racie et al. 2004), to which versican is closely related, it is possibly a versican-cleaving protease as well. It is also likely that additional ADAMTS proteases also cleave versican, or that versican is cleaved by members of other protease classes.

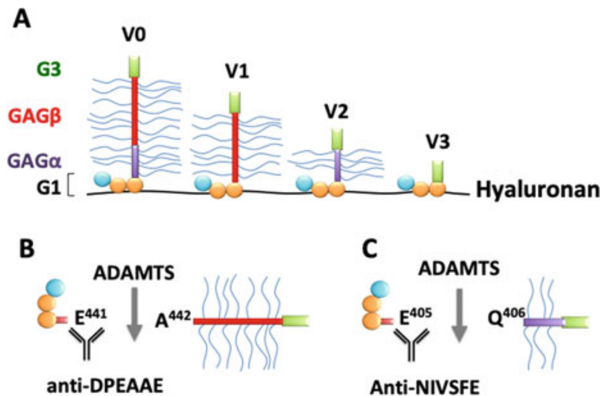

Fig. 3.1 Versican isoforms and ADAMTS processing sites in the versican core proteins. (**a**) Splice isoforms of versican are indicated to show specific inclusion or exclusion of alternatively spliced exons encoding the GAGα and GAGβ domains. Each isoform is bound to hyaluronan (black line) via its G1 domain. (**b**, **c**) Cartoons depicting the major ADAMTS cleavage sites in the GAGβ (**b**) and GAGα (**c**) domains and the neo-epitope antibodies used to detect these cleavages

Versican shares the overall structure of the large aggregating proteoglycans, having N-terminal and C-terminal globular domains, with a CS-chain bearing region between them. This interglobular region is of variable length, depending on inclusion of one or both of two large alternatively spliced exons encoding CS-bearing regions termed GAGα (encoded by exon 7) and GAGβ (encoded by exon 8), respectively (Zimmermann and Ruoslahti 1989; Dours-Zimmermann and Zimmermann 1994) (Fig. 3.1a). Of the 4 resulting splice isoforms, V3 lacks both exons and thus has no GAG chains, whereas V0 contains both exons (estimated 17–23 CS chains). V1 (estimated to have 12–15 CS chains) and V2 (estimated to have 5–8 CS chains) contain exon 8 or exon 7, respectively. V2 appears to be specific for the nervous system (Schmalfeldt et al. 1998), whereas V1 and V0 are most abundant outside the nervous system and are expressed by a variety of cells including limb bud mesenchyme, differentiating cardiomyocytes, fibroblasts, and smooth muscle cells of various organs such as blood vessels and the uterus (Bode-Lesniewska et al. 1996). Thus, proteolytic turnover of V1 and V0 is thought to be more relevant to reproduction and development than turnover of V2. The exon 8-encoded GAGβ region contains a sequence homologous to the sequence context of major ADAMTS processing sites in aggrecan and brevican (Glu^{374}-Ala^{375} and Glu^{395}-Ser^{396}, respectively; human sequence enumeration) (Sandy et al. 2001). Accordingly, the Glu^{441}-Ala^{442} peptide bond of versican, which is located near the N-terminus of the GAGβ domain (human V1 sequence enumeration; corresponding position in V0 is Glu^{1428}-Ala^{1429}) was predicted by John Sandy as a cleavage site for ADAMTS proteases (Sandy et al. 2001). His group generated a neo-epitope antibody to the corresponding C-terminal $DPEAAE^{441}$ peptide and showed that versican fragments resulting from treatment with ADAMTS1 and ADAMTS4 were reactive with this antibody (Sandy et al. 2001) (Fig. 3.1b). Anti-DPEAAE is now recognized as a

powerful and specific tool for investigating versican processing by ADAMTS proteases. Another cleavage site, Glu^{405}-Gln^{406}, occurring in the GAGα domain (present in V2 and V0 isoforms), is similarly located near its N-terminus. Cleavage at this site is detected by a different neo-epitope antibody, anti-NIVSFE (Fig. 3.1c), but is less well characterized (Westling et al. 2004). Like aggrecan, which is cleaved at several sites within the core protein by ADAMTS proteases (Tortorella et al. 2000), it is likely that there are additional ADAMTS cleavage sites in the versican core protein (Jonsson-Rylander et al. 2005), but this possibility has not been vigorously investigated. Furthermore, it is likely that several other proteases such as MMPs may be involved in versican turnover. Despite this breadth of possibilities and potential compensation by other protease classes, there is nevertheless strong genetic evidence that ADAMTS proteases are specifically required for versican turnover. In addition to association of *Vcan* expression with that of ADAMTS genes and appearance of the 70-kDa anti-DPEAAE reactive fragment during embryonic stem cell differentiation into various lineages in embryoid bodies (Shukla et al. 2010), inactivation of specific ADAMTS genes in mice has led to defects in reproduction and morphogenesis that are consistently associated with reduced versican cleavage (Nandadasa et al. 2014). In contrast, mouse MMP mutants are not associated with such defects. Thus, it can be concluded that ADAMTS proteolysis of versican, albeit at Glu^{441}-Ala^{442} or other, yet to be defined sites, is of considerable significance in reproduction and development.

3.3 Versican Turnover During Ovulation and Role of ADAMTS Proteases in Fertility

Prior to and at ovulation, the oocyte is surrounded by a layer of specialized granulosa cells and ECM called the cumulus oophorus, and is known as the cumulus oocyte complex (COC) (Curry 2010). Versican-HA aggregates are a major component of COC ECM (Dunning et al. 2015). During fertilization, sperm hyaluronidase degrades this ECM for access to the oocyte membrane. At ovulation, the mature ovarian follicle ruptures and releases the COC into the peritoneal cavity and then travels to the fallopian tube, where fertilization occurs. Ovulation is a dynamic process orchestrated by several hormones (Curry 2010). Primordial follicles mature under the influence of estrogen and progesterone: first follicle stimulating hormone and, subsequently, a pre-ovulatory surge of luteinizing hormone (LH) lead to progesterone upregulation and follicular growth and maturation, a key component of which is expansion of the cumulus cells surrounding the oocyte. Successful expansion of the COC ECM is essential for ovulation and ultimately for fertilization, and is achieved by increased production of HA and proteoglycans. Dissolution of the connective tissue and cellular layers at the follicular apex and ovarian surface are necessary for release of the oocyte (Russell et al. 2003). Furthermore, cumulus cell growth is accompanied by increased proteolysis of COC ECM (Russell et al. 2003).

These changes in the COC are crucial for priming the oocyte and transporting it to the oviduct prior to fertilization. ADAMTS1 is induced by the LH surge-dependent increase in progesterone and progesterone receptor expression in mural granulosa cells of mice (Espey et al. 2000; Robker et al. 2000). After secretion, mature ADAMTS1 associates with the COC-ECM (Russell et al. 2003; Doyle et al. 2004), consistent with observations that ADAMTS1 has a strong affinity for ECM, and specifically, negatively charged molecules such as heparin (Kuno and Matsushima 1998; Kuno et al. 1999). Human chorionic gonadotropin (hCG) administration to women followed by analysis of harvested follicles identified upregulation of *ADAMTS1* and *ADAMTS9* mRNA in granulosa cells, but not thecal cells (Rosewell et al. 2015). ADAMTS1 cleaves versican both within the COC ECM and follicular wall and is vital for ensuring cumulus cell expansion and follicular wall remodeling in mice (Mittaz et al. 2004).

A number of proteases were implicated by their association with COC ECM turnover, but few were unequivocally implicated until the discovery that female *Adamts1*$^{-/-}$ mice were subfertile due to impaired ovulation, with the mature oocytes found to be trapped in the follicles instead of being released (Brown et al. 2006; Shozu et al. 2005). The ovulation rate in *Adamts1*$^{-/-}$ mice was reduced by 77% and fertilization of ovulated oocytes was reduced a further 63% (Mittaz et al. 2004). The follicles lacked vascular invagination in their basal region where versican cleavage was significantly reduced compared to wild-type follicles (Brown et al. 2010). This was the first evidence that processing of versican by ADAMTS1 was involved in ovulating follicle remodeling. Furthermore, both versican and hyaluronan were abnormally located in the COC during expansion of its matrix, and versican persisted beyond the expected time of fertilization in the mutant mice, whereas it underwent proteolysis and was cleared from control COC (Brown et al. 2010). Thus, ADAMTS1 mediates both the turnover of proteoglycans in the COC ECM and the remodeling of the follicular wall that is essential for ovulation. *ADAMTS1* expression levels correlate with follicle size, cumulus cell number, and fertilization capacity of the human oocyte (Yung et al. 2010). Moreover, in women with polycystic ovary syndrome, *ADAMTS1* mRNA levels were lower in granulosa cells isolated from their ovarian follicles and the levels were closely related to oocyte recovery, maturity, and fertilization rate (Xiao et al. 2014).

Adamts4 and *Adamts5* mRNA expression patterns overlap partially with that of *Adamts1* (Richards et al. 2005), and these proteases may contribute to ovarian remodeling, but the respective mouse mutants are not reported to be subfertile or infertile. Recently, *Adamts9* inactivation in zebrafish was reported to be associated with reduction of female offspring, failure of ovarian development in the few female progeny of the mutants that were observed, along with lack of ovulation, and unexpectedly, association of spermatozoa with the abnormal ovaries identified in these females (Carter et al. 2019). The underlying mechanisms of this effect and its relationship to versican processing are unknown.

3.4 ADAMTS9 Is Essential for Myometrial Activation and Parturition

The uterus has two major components, an inner lining called the endometrium, and the outer contractile myometrium, whose function is to contract rhythmically and forcefully to expel the fetus and afterbirth. In preparation for this role, the myometrium grows steadily in mass through pregnancy, accumulating collagen-rich ECM and smooth muscle cells (SMC). Toward the end of pregnancy, the myometrial SMCs attain a heightened state of differentiation termed "activation" that renders them highly contractile and responsive to parturition hormones (Shynlova et al. 2009; Taggart and Morgan 2007). These changes include higher expression of contractile proteins, connexin-43, and oxytocin and prostaglandin F2a receptors, each providing specific and desired characteristics to the activated myometrium. At parturition, synchronous SMC contraction initiated by oxytocin and prostaglandin F is supported by heightened cell–cell communication provided by connexin-43 gap junctions and robust SMC contraction is transmitted by a fibrillar collagen network, which also provides structural integrity to the uterus. *Adamts9* expression was observed in both gravid and non-gravid mouse myometrium, whereas other versican-degrading ADAMTS proteases were not specifically expressed therein (Mead et al. 2018).

Global *Adamts9* inactivation in mice is lethal at the time of gastrulation, so its function in myometrial and other SMC populations could not be studied in these mice (Benz et al. 2016; Enomoto et al. 2010). Conditional *Adamts9* inactivation in smooth muscle cells using *Tagln*-Cre led to failure of parturition in mice as a result of an impaired myometrial activation process (Mead et al. 2018). Specifically, in the absence of ADAMTS9, major contractile proteins, prostaglandin and oxytocin receptors, and gap junction expression were all attenuated (Fig. 3.2) (Mead et al. 2018). These defects were associated with reduction in versican processing and accumulation of versican around SMC (Mead et al. 2018). Ultrastructurally, *Adamts9*-deficient myometrium showed the presence of abundant amorphous ECM, which is typical of a proteoglycan-rich matrix (Mead et al. 2018). In the presence of this abundant proteoglycan-rich ECM, SMC may be unable to make sufficient cell–cell contact to form gap junctions and may also become disconnected from the collagen network that transmits their contraction.

Indeed, *Adamts9* knockdown in cultured uterine SMC also led to accumulation of pericellular ECM, impaired focal adhesion formation, and was associated with similar impaired differentiation observed in vivo (Fig. 3.2) (Mead et al. 2018). We postulated that accumulation of uncleaved versican in the absence of ADAMTS9 impaired focal adhesion formation as a primary mechanism, and it is likely that the lack of adhesive inputs to the cytoskeleton led to an altered transcriptional program. Indeed, changes in cell shape as well as nuclear shape were identified, and uterine SMC transfected with *ADAMTS9* siRNA showed a remarkable tendency to round up and lose attachment to the underlying substratum, yet adhered to each other (Mead et al. 2018). The likelihood that versican is a major ADAMTS9 substrate involved in

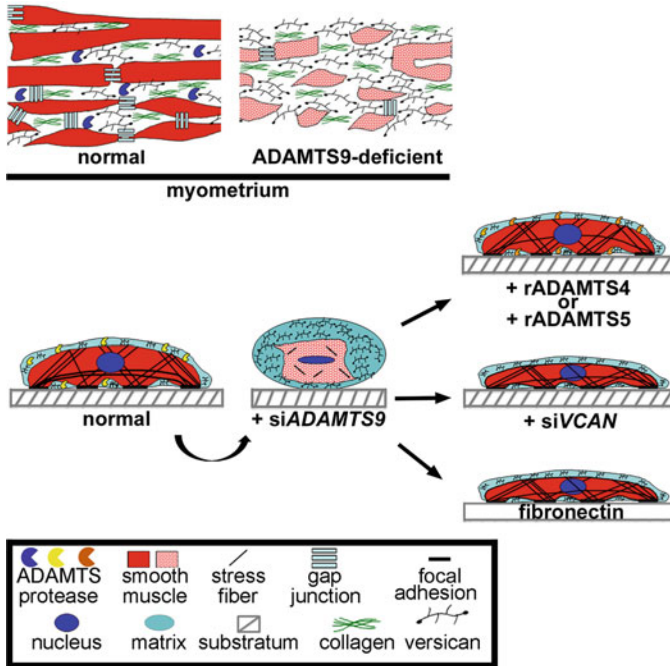

Fig. 3.2 ADAMTS9-regulated pericellular versican processing regulates focal adhesion formation and myometrial activation. The contrast between activation of normal and ADAMTS9-deficient myometrium is shown at the top, as explained by the key at the bottom of the figure. The center panel summarizes the cellular mechanism of ADAMTS9, showing that ADAMTS9 knockdown leads to accumulation of pericellular versican and cell rounding because of interference with focal adhesion formation. This deleterious effect was reversed by treating these cells with recombinant ADAMTS4 or ADAMTS5, or concurrent knockdown of *VCAN*. This figure was previously published in: Mead TJ, Du Y, Nelson CM, Gueye N-A, Drazba J, Dancevic CM, Vankemmelbeke M, Buttle DJ, Apte SS. ADAMTS9-Regulated Pericellular Matrix Dynamics Governs Focal Adhesion-Dependent Smooth Muscle Differentiation. *Cell Reports.*2018, 23; 2, 485–498 and is reproduced with the permission of the publisher Cell Press/Elsevier

uterine SMC dedifferentiation was supported by restoration of differentiation by concomitant *VCAN* and *ADAMTS9* knockdown in uterine SMC, or by addition of exogenous ADAMTS4 or ADAMTS5 to provide a versicanase activity (Fig. 3.2) (Mead et al. 2018). Thus, reduced versican turnover and consequent accumulation in pericellular ECM can have a dramatic effect on cell phenotypes via organization of the cytoskeleton (Fig. 3.2) and echoed prior work which had found that versican accumulation in dermal fibroblast pericellular ECM, such as by *Adamts5* inactivation or *Vcan* overexpression, led to their transition to myofibroblasts (Carthy et al. 2015; Hattori et al. 2011). Recently, we have generated mice with combined conditional deletion of *Adamts9* in smooth muscle cells and *Vcan* haploinsufficiency, and interestingly, these mice give birth without any problems (Mead et al. manuscript in preparation). In addition, we and others (Islam et al. 2019)

generated transgenic mice with mutations of the Glu^{441}-Ala^{442} site to render it uncleavable; these mice apparently give birth without impairment. This suggests that while versican cleavage at the Glu^{441}-Ala^{442} site is a useful reporter of ADAMTS activity, other cleavage sites in versican probably ensure that it is turned over adequately despite the mutation. Indeed, aggrecan processing by ADAMTS proteases, which has been researched more extensively than versican processing, occurs at numerous sites (Tortorella et al. 2000). None of the other versican-degrading ADAMTS proteases are expressed in the myometrium as strongly as ADAMTS9 (Mead et al. 2018), and their knockouts do not apparently impair parturition. Hence, ADAMTS9 has a unique role in regulation of the versican content of gravid myometrium.

3.5 ADAMTS Proteolysis of Versican in the Maternal-Fetal Interface

The fetal–maternal interface includes the placenta, fetal membranes, and umbilical cord. Working together, they assure nutrient, waste, and gas exchange in the growing embryo and provide immunologic protection. These tissues grow steadily through gestation and therefore undergo continuous ECM remodeling in a similar fashion as the embryo. ADAMTS proteolysis of versican is implicated in proper development of the umbilical cord and yolk sac. *Adamts9* is expressed in the Wharton's jelly mesenchyme, the adventitial cells of umbilical arteries, and the endothelium of the umbilical vein (Nandadasa et al. 2015). Although global *Adamts9* mutants die by 7.5 days of gestation, hypomorphic *Adamts9* mutants (*Adamts9*$^{Gt/Gt}$) survive until 14.5 days of gestation (Nandadasa et al. 2015). These mice have a defect in umbilical cord development that manifests as short cords, and is associated with intrauterine growth retardation (Nandadasa et al. 2015). Versican accumulation was seen between the smooth muscle cells of the umbilical artery and in the surrounding cells, manifesting as accumulation of an amorphous interstitial extracellular matrix (i.e., without discernible macromolecular complexes such as fibers or fibrils), which is typical of a proteoglycan-rich ECM (Nandadasa et al. 2015).

In addition to the short umbilical cord, *Adamts9*$^{Gt/Gt}$ mutants have anomalous umbilical artery development. In immature blood vessels, SMC are oriented parallel to the vessel axis whereas mature blood vessels have circumferentially oriented SMC. Orthogonal re-orientation from longitudinal to circumferential occurs as a critical morphogenetic step in vascular development (Greif et al. 2012). Accumulation of uncleaved versican in the smooth muscle cells of the umbilical arterial wall in the absence of ADAMTS9 was associated with failure of orthogonal rotation of these cells to assume the correct circumferential orientation around the arterial lumen (Nandadasa et al. 2015). Thus, ADAMTS9 secreted by umbilical cord adventitial fibroblasts and Wharton's jelly mesenchymal cells acted non-autonomously in

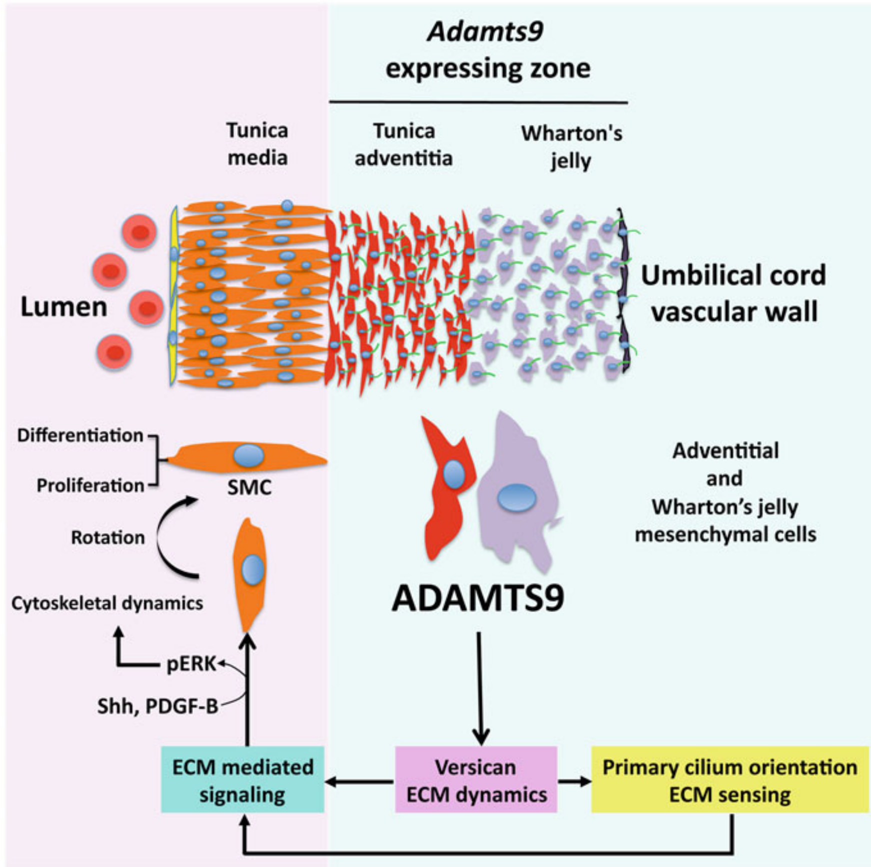

Fig. 3.3 ADAMTS9-mediated versican processing regulates umbilical cord vascular smooth muscle differentiation and rotation. The upper part of the figure is a cartoon depicting the arrangement and orientation of the major cell populations in the umbilical artery wall. The lower half of the figure summarizes the effect of ADAMTS9 expressed by smoooth muscle cells and adventitial cells on versican dynamics, which is postulated to lead to ECM-mediated control of sonic hedgehog (Shh) and platelet-derived growth factor B (PDGF-B)-ERK signaling, correct primary cilium orientation, and smooth muscle cell differentiation. This figure was previously published in: Nandadasa, S, Nelson, C.M., Apte, S.S. ADAMTS9 mediated extracellular matrix dynamics regulates umbilical cord vascular smooth muscle differentiation and rotation. *Cell Reports* 2015, 11:1519–28 and is reproduced with the permission of the publisher Cell Press/ Elsevier

development of the vascular wall SMC. These cells had abnormal orientation of their primary cilium, an organelle essential for Shh signaling, which was impaired in the mutant umbilical arterial wall (Nandadasa et al. 2015). The combination of versican accumulation and abnormal ciliary orientation is thought to be the mechanism underlying anomalous vascular development in these mutant umbilical cords (Fig. 3.3). Whether versican accumulation directly affects the cilium orientation

has not been resolved. The other extra-embryonic tissue found to be abnormal in these mutants was the yolk sac. *Adamts9*, but not its homolog *Adamts20,* is expressed in yolk sac mesoderm (Nandadasa et al. 2019). Similar to the umbilical cord, the *Adamts9*$^{Gt/Gt}$ yolk sac demonstrated versican accumulation in the mesoderm layer, as well as abnormal cilia in the yolk sac mesothelium and impaired hedgehog signaling (Nandadasa et al. 2019).

3.6 ADAMTS Proteolysis of Versican During Morphogenesis

Impaired versican processing is observed during morphogenesis in several ADAMTS mouse mutants. Following on the realization that ADAMTS9 and ADAMTS20 were highly conserved, it was of interest to determine whether mice with combined deficiency would disclose cooperative functions for these proteases. However, early lethality of *Adamts9*$^{-/-}$ embryos precluded the possibility of obtaining this information from double knockout embryos, since these would not survive past gastrulation. Therefore, Enomoto et al. (2010) generated a partial combined knockout, i.e., one copy of *Adamts9* was deleted in mice homozygous for the *Adamts20*^{Bt} allele, a spontaneous ADAMTS20 loss-of-function mutant (Rao et al. 2003). Similarly, *Adamts20*$^{Bt/Bt}$;*Adamts9*$^{Gt/+}$ embryos were produced (Nandadasa et al. 2015). Both sets of combined mutants died at birth with cleft palate and reduced skin pigmentation, identifying a cooperative role in palatogenesis and melanoblast colonization of skin, respectively (Enomoto et al. 2010; Nakatani et al. 2006). Reduced versican processing was observed in the palate and skin of *Adamts20*$^{Bt/Bt}$; *Adamts9*$^{-/+}$ and *Adamts20*$^{Bt/Bt}$ mutants, respectively (Enomoto et al. 2010; Silver et al. 2008).

Adamts20$^{Bt/Bt}$;*Adamts9*$^{Gt/Gt}$ embryos, like *Adamts9*$^{Gt/Gt}$ embryos, survive to 14.5 days of gestation, but manifest extremely severe and widespread anomalies (Nandadasa et al. 2019). The observed defects included exencephaly (neural overgrowth with failure of neural tube closure), failure of midline facial fusion, heterotaxy, and renal, lung, and cardiac dysgenesis (Nandadasa et al. 2019). Detailed analysis of neural tube development identified dramatic accumulation of chondroitin sulfate and versican as well as reduced versican proteolysis in the neural floor plate. Unexpectedly, ciliogenesis, i.e., formation of the primary cilium, was severely impaired (Nandadasa et al. 2019). Targeted inactivation of *ADAMTS9* in RPE-1 cells using CRISPR-Cas9 unequivocally established the role of ADAMTS9 in ciliogenesis (Nandadasa et al. 2019). Ciliogenesis was restored in the *ADAMTS9* mutant RPE-1 cells by transfection of catalytically active ADAMTS9 and ADAMTS20, but not by transfection of the inactive mutants of these proteases (Nandadasa et al. 2019). ADAMTS9 inactivation in RPE-1 cells (which naturally lack *ADAMTS20* expression) also led to accumulation of versican in their ECM, but versican overexpression in RPE-1 cells did not result in defective ciliogenesis

(Nandadasa et al. 2019). ADAMTS9 cleaves fibronectin (Wang et al. 2019), which is also known to accumulate in *Adamts9* mutants (Nandadasa et al. 2015, 2019; Dubail et al. 2016). Thus, ADAMTS9 and ADAMTS20 are major contributors to versican turnover during the organogenesis period (9.5 to 13.5 days of mouse gestation).

ADAMTS proteases are implicated in versican proteolysis during several aspects of cardiac development. Versican is itself necessary for cardiac development, and *Vcan*$^{hdf/hdf}$ mutant mouse embryos do not survive past 10.5 days of gestation (Mjaatvedt et al. 1998); indeed, according to recent work from our laboratory, versican is also essential for formation of the vasculature and blood (Nandadasa, S., O'Donnell, A., Midura, R.J., Apte, S.S.,*The versican-hyaluronan complex provides an essential extracellular matrix niche for Flk1+ precursors in vasculogenesis and primitive hematopoiesis,* https://www.biorxiv.org/content/10.1101/753418v1).

Hence, versican is crucial for development of the entire cardiovascular system, and its proteolytic turnover during cardiac development is of commensurate importance. Versican is a major component of cardiac jelly, an amorphous matrix secreted by developing cardiomyocytes during myocardiogenesis (Henderson and Copp 1998). Once a sufficient number of cardiomyocytes have been formed and their maturation begins, *Vcan* expression is downregulated. Cardiac jelly rapidly undergoes regression by the action of ADAMTS proteases such as ADAMTS1 and ADAMTS5, which are secreted by the endocardial and myocardial cells, allowing a compact myocardium to be formed by 12.5 days of gestation (Del Monte-Nieto et al. 2018). Areas of cardiac jelly persist around the ventricular trabeculae until 14 days of gestation, and these too eventually undergo ADAMTS-mediated digestion (Del Monte-Nieto et al. 2018). Specifically, de-repression of endocardial ADAMTS1 expression by inactivation of the transcriptional repressor BRG-1 resulted in premature resorption of cardiac jelly and reduced myocardial trabeculation (Stankunas et al. 2008). A cardiac jelly-like ECM is also present in the endocardial cushions which form the heart valves (Henderson and Copp 1998) and sculpting of the cushions into thin valve leaflets is associated with *Adamts1*, *Adamts5,* and *Adamts9* expression (Dupuis et al. 2011; Kern et al. 2006; McCulloch et al. 2009a). Valve defects associated with versican accumulation have been reported in *Adamts5* and *Adamts9* mutant mice (Dupuis et al. 2011, 2013; Kern et al. 2006, 2010).

One of the most dramatic and insightful effects of ADAMTS proteolysis of versican occurs during interdigital web regression (McCulloch et al. 2009b). Mammalian embryos have interdigital webs until completion of limb patterning, and these then undergo rapid resorption. For decades, this process was studied exclusively in the context of programmed cell death, with little consideration given to the fate and role of ECM turnover in the process. *Vcan* is strongly expressed in the webs around 12.5 days of gestation and shortly after, i.e., just prior to and during web regression, *Adamts1, Adamts5, Adamts9*, and *Adamts20* expression are all highly upregulated. Analysis of mice with pair-wise deletion of *Adamts5, Adamts9,* and *Adamts20* or limb-specific conditional deletion of *Adamts9* demonstrated that they had defective web regression and failure of apoptosis in the interdigit mesenchyme (McCulloch

et al. 2009b; Dubail et al. 2014). Introduction of *Vcan* haploinsufficiency in *Adamts20*$^{Bt/Bt}$ mice worsened soft tissue syndactyly in these mutants, suggesting an essential role for versican or a versican fragment in interdigit apoptosis (McCulloch et al. 2009b). Indeed, treatment of the unresorbed webs of *Adamts5*$^{-/-}$*;Adamts20*$^{Bt/Bt}$ limbs ex vivo with the G1-DPEAAE^{441} fragment, henceforth named versikine, induced cell death (McCulloch et al. 2009b). This work strongly suggested that interdigit matrix proteolysis by ADAMTS proteases, and specifically of versican, was essential for subsequent apoptosis. Thus, ECM and cell regression occurred coordinately to ensure completion of this crucial morphogenetic mechanism.

3.7 Summary

Versican is a major component of the provisional ECM of the mammalian embryo, and ADAMTS proteases have a dominant role in its proteolysis during the embryonic period and beyond. The investigations carried out to date suggest that in addition to removal of versican, ADAMTS proteases can also generate a biologically active versican fragment named versikine. Another important concept to emerge from these studies is that ADAMTS proteases cooperate during versican proteolysis. Finally, remodeling of pericellular versican has emerged as an important mechanism that regulates focal adhesion formation, cytoskeletal assembly, and the correct differentiation of smooth muscle cells and fibroblasts in specific tissue contexts.

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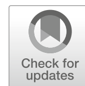
Chapter 4
Use of Chondroitin Sulphate to Aid In Vitro Stem Cell Differentiation



Brooke Farrugia, Anthony J. Hayes, and James Melrose

Abstract Chondroitin sulphate (CS) has essential roles to play in the stem cell niche with regard to stem cell differentiation and the attainment of pluripotency. The sulphation status of CS is an important determinant of its functional properties, and this parameter has been used in innovative bioscaffold design to direct the differentiation of stem cells in culture. The intrinsic charge properties of CS determine its interactive properties with extracellular matrix (ECM) components, growth factors, cytokines, and morphogens regulating the growth and differentiation of the resident stem cell populations. Stem cells express cell surface proteoglycans decorated with CS sulphation motifs identified by the monoclonal antibodies 4-C-3, 7-D-4 and 3-B-3(−). These CS sulphation motifs occur at many important centres/ interfaces of growth and differentiation in tissue morphogenesis during the development of a wide range of connective tissues. The 3-B-3(−) sulphation epitope is a non-reducing terminal CS epitope generated by HYAL4 produced by mast cells. Stem cells also generate the 3-B-3(−) epitope. A greater understanding of the interactive properties of the aforementioned CS sulphation motifs with ECM

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components, growth factors and morphogenetic proteins will be insightful as to how stem cells control and interact with their tissue environment to regulate tissue development and tissue homeostasis.

Abbreviations

Cbf-β	Core binding factor beta
CS-PG	Chondroitin sulphate proteoglycan
GAG	Glycosaminoglycan
BMPs	Bone morphogenetic protein
CS	Chondroitin sulphate
DS	Dermatan sulphate
ECM	Extracellular matrix
FGF	Fibroblast growth factor
HA	Hyaluronan
Hyal4	Hyaluronidase-4
Hh	Hedgehog (protein)
HS	Heparan sulphate
HS-PG	Heparan sulphate proteoglycan
Ihh	Indian hedgehog
MK	Midkine
PCM	Pericellular matrix
PEG	Poly ethylene glycol
PG	Proteoglycan
PTH	Parathyroid hormone
PTHrP	Parathyroid hormone-related protein
PTHrPR	Parathyroid hormone-related protein receptor
PTN	Pleiotrophin
qRTPCR	Quantitive reverse transcription-polymerase chain reaction
Runx2	Runt related transcription factor 2
SLRP	Small leucine-rich repeat proteoglycan
Wnt	the term ***Wnt*** is derived from a mouse proto-oncogene (Int1, integration-1 protein) and the *Wingless* protein originally identified in *Drosophila.* The **Wnt** term is a condensation of the ***int*** and ***Wg*** protein terms and stands for *Wingless-related integration site*
3-B-3(−)	a non-reducing terminal CS sulphation motif identified by MAb 3-B-3 in the absence of chondroitinase ABC digestion
2-B-6 (−)	a non-reducing terminal CS sulphation motif identified by MAb 2-B-6 in the absence of chondroitinase ABC digestion
3-B-3(+)	linkage region, terminal C-6-S disaccharide stub 3-B-3 sulphation motif generated by chondroitinase ABC digestion of the CS chain
2-B-6 (+)	linkage region, terminal C-4-S disaccharide stub 2-B-6 sulphation motif generated by chondroitinase ABC digestion of the CS chain

A76	Monoclonal antibody to perlecan domain-1
A7L6	Monoclonal antibody to perlecan domain IV
3A4	Monoclonal antibody to lubricin, PRG4
12C5	Monoclonal antibody to versican G1 HA binding domain
4-C-3	Monoclonal antibody to a native CS sulphation motif
7-D-4	Monoclonal antibody to a native CS sulphation motif
6-B-4	Monoclonal antibody to a linear amino acid sequence 394EPEEPFTFAPEI406 in the interglobular domain of human and bovine aggrecan in intact and protease digested aggrecan

4.1 Introduction

Chondroitin sulphate (CS) is a glycosaminoglycan (GAG) composed of glycosidically linked β1-3 and β1-4 D-glucuronic acid and N-acetyl galactosamine residues which can be *O*-sulphated at the C2, C4 or C6 positions. The D-glucuronic acid component of CS may also be epimerized to αL-Iduronic acid in the related GAG, dermatan sulphate (DS), leading to a considerable degree of structural biodiversity with over one thousand pentasaccharide sequences possible in CS/DS (Cummings 2009). These GAG structures interact with a large range of growth factors, cytokines, morphogenetic factors, enzymes and inhibitors, which can promote connective tissue remodelling, cellular differentiation and proliferation in tissue development, health and disease (Hayes et al. 2018a; Hayes and Melrose 2018; Melrose 2016, 2017; Melrose et al. 2016; Caterson 2012; Whitelock et al. 2008).

The 3-B-3(−), 7-D-4 and 4-C-3 CS sulphation motifs in CS chains (Caterson 2012) were identified in intact native CS chains following partial depolymerization using chondroitinase ABC (Fig. 4.1c) (Caterson 2012). All of these epitopes are sensitive to chondroitinase ABC, 3-B-3(−) reactivity is initially removed from the CS chain, followed by 4-C-3 and 7-D-4 epitopes. Complete enzymatic digestion of the CS chain using chondroitinase ABC generates unsaturated 3-B-3(+) and 2-B-6 (+) stub disaccharide epitopes at the reducing terminus attached directly to the tetrasaccharide linker region of the CS to proteoglycan (PG) core proteins (Fig. 4.1c). These stub epitopes should not be confused with the 3-B-3(−) and 2-B-6(−) epitopes, which are terminal epitopes at the non-reducing terminus of the GAG chain generated by HYAL 4 (Fig. 4.1c).

The mast cell PGs serglycin and perlecan have been reported to display 2-B-6(−) epitopes on their CS chains (Farrugia et al. 2016a). Like 3-B-3(−), 2-B-6(−) is not generated by chondroitinase ABC digestion, and until relatively recently, it was not known what mammalian enzyme generated these epitopes. Chondroitinase ABC is a bacterial eliminase that cleaves the glycosidic linkages between glucuronic acid and galactosamine mainly yielding disaccharide products containing unsaturated glucuronic acid moieties, which absorb at 232 nm and thus can be measured by UV spectroscopy. The 2-B-6(−) epitope has previously been reported in

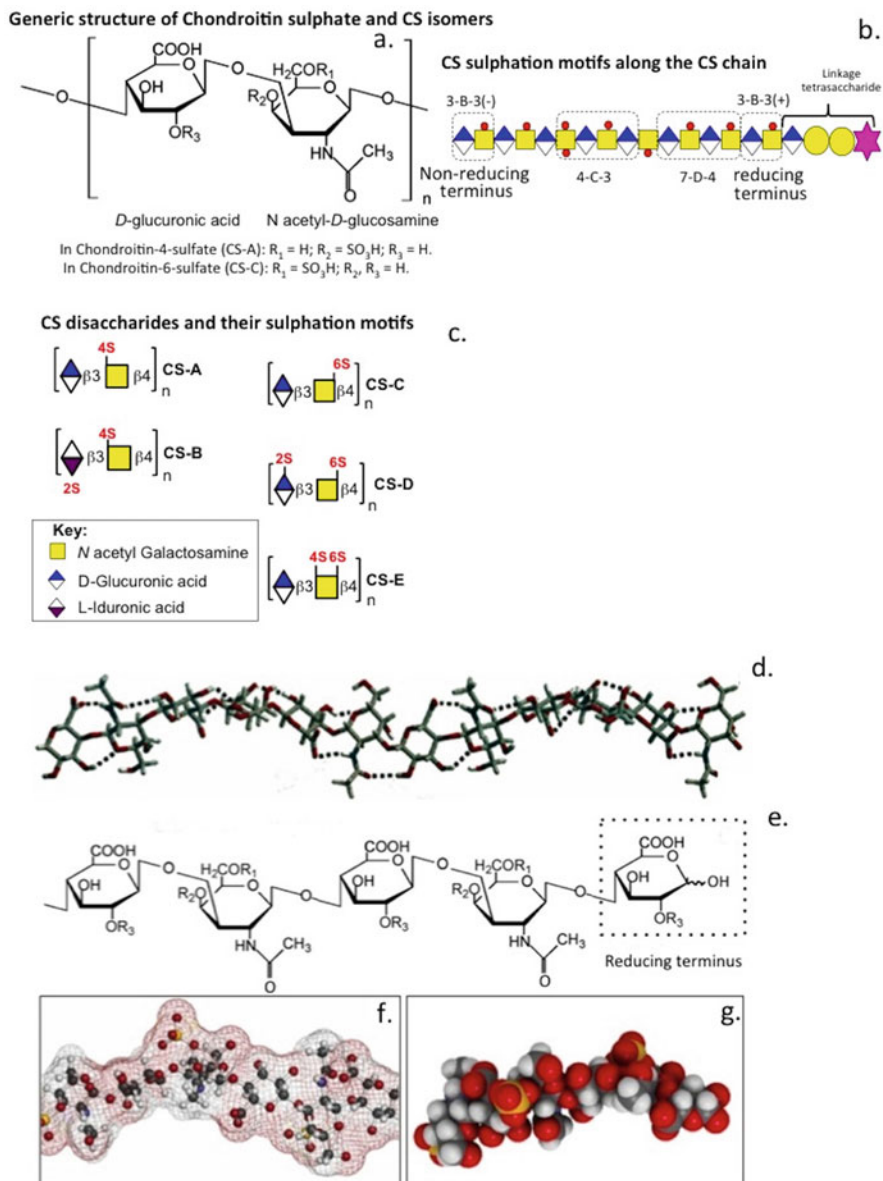

Fig. 4.1 Generic structure of chondroitin sulphate (**a**), CS disaccharides of CS isoforms (**b**) and CS sulphation motifs identified by antibodies, 3-B-3, 7-D-4 and 4-C-3 (**c**). Structural depiction of the helical structure of CS in stick (**d**) chemical (**e**), space filling (**f**) and globe models (**g**)

osteoarthritic cartilage (Asari et al. 1996). Members of the hyaluronidase (HYAL) family, HYAL-1 and HYAL-4, which are CS hydrolases cleaving CS in the $\beta1{\rightarrow}4$ disaccharide glycosidic linkage (Yamada et al. 2009), are also able to generate 2-B-6

(−) and 3-B-3(−) epitopes (Farrugia et al. 2016b, 2019). The 2-B-6(−) and 3-B-3 (−) epitopes and production of HYAL-4 by mast cells are associated with connective tissue remodelling in inflammatory conditions (Farrugia et al. 2019). The findings of the present study also suggest that chondroprogenitor cells may also express HYAL-4.

CS occurs in a number of sub-classes (Fig. 4.1), including the high charge density CS-D and E and the lesser charged CS-A, B and C. CS-D and E are enriched in the brain in the GAG side chains of PGs such as appican, syndecan-1, syndecan-4, neuroglycan-C and phosphacan (Deepa et al. 2004; Shuo et al. 2004; Tsuchida et al. 2001). CS-A, B, C are found as side chains in the brain perineural net forming hyalectan or lectican PG family, which includes brevican, neurocan, aggrecan and versican (Yamaguchi 2000; Kwok et al. 2008; Pantazopoulos et al. 2015; Rhodes and Fawcett 2004; Soleman et al. 2013). CS-A and C are also side-chain components of aggrecan, the major space-filling CS-proteoglycan of cartilaginous matrices which provides weight-bearing properties and tissue hydration through its ability to form macromolecular ternary complexes with hyaluronan (HA) and link protein which entrap water within tissues (Kiani et al. 2002). Decorin and biglycan, members of the small leucine repeat PG (SLRP) family, also contain CS-A, CS-B and CS-C side chains, although this varies with tissue type (Chen et al. 2017; Schaefer and Iozzo 2008; Waddington et al. 2003). Decorin and biglycan in bone are substituted with CS-A and CS-C, while in tensional and weight-bearing tissues such as cartilage and tendon, they may also be substituted with CS-B. CS chains are normally drawn as linear structures however, on closer inspection of stick, space-filling and globe model representations of the CS chain, it becomes obvious that the CS chain is actually a helical structure that allows the optimal distribution of mutually repulsive bulky sulphate groups which are so important for the functional properties of CS (Fig. 4.1d–g).

4.2 CS Sulphation Motifs Regulate Cell Behaviour, Can They Be Used to Promote Tissue Repair?

The sulphation motifs of CS-proteoglycans have pivotal regulatory roles in cell migration and tissue morphogenesis (Ishii and Maeda 2008; Malmstrom et al. 2012; Zimmer et al. 2010). Novel CS sulphation sequences occur in the functionally distinct layers of skin (Sorrell et al. 1990); are associated with the long bone growth plates in endochondral ossification and occur at important growth zones in the developing intervertebral disc, diarthrodial joints and tendon (Caterson et al. 1990; Hayes et al. 2001). During lymphopoeisis, CS sulphation motifs are differentially modified at sites of B-cell differentiation and maturation (Caterson et al. 1990; Sorrell et al. 1988) and in the brain CS sulfation plays an important role in neurite outgrowth, synaptic plasticity and neurological development (Rhodes and Fawcett 2004). CS also has indispensable roles to play in stem cell differentiation and

attainment of pluripotency (Izumikawa et al. 2014). Accumulated evidence, therefore, points to CS sulphation motifs having critical roles in cell interactions, in cell differentiation, proliferation and matrix assembly processes.

4.2.1 Evolution of GAGs as Cellular Mediator Molecules

GAGs are ancient molecules that have evolved over 500 million years of vertebrate and invertebrate evolution (Yamada et al. 2011). Phylogenetic selection processes have resulted in the predominance of specific GAG structures through millions of years of positive evolutionary selection pressure and the generation of a vast library of heterogeneous glycan structures. GAGs have important roles to play in a multitude of essential physiological processes required for cellular survival. This explains why GAGs persist to the present day despite the significant genetic investment a cell must make in the multiple biosynthetic and modification enzymes required to assemble these intricate structures. Virtually every cell has a dense surrounding glycocalyx that consists of a mixture of glycoproteins, PGs and glycolipids, which provide a protective and interactive barrier (Furukawa et al. 2016). CS is a prominent GAG component that decorates many of these molecules and is the most abundant GAG in the human body. The glycocalyx is an extension of the cell into its external environment and also provides an inter-connection with neighbouring cells. The glycocalyx acts as a biosensory structure that communicates changes in the extracellular environment to the cells within it. Cells interpret this information and respond to changes in their microenvironment by modulating the biosynthesis of extracellular matrix (ECM) components to maintain a protective functional ECM and a homeostatic balance in tissue composition. The endothelial glycocalyx has mechanosensory shear and compression responsive capability, which regulates mechano-transductive effects on endothelial cell signalling and vascular permeability important in the nutrition and development of tissues (Furukawa et al. 2016; Curry and Adamson 2012; Dawson 2014; Tarbell and Ebong 2008; Tarbell et al. 2014). In the brain, CS exerts an inhibitory regulatory effect on the outgrowth of dendrites in neurons. The glycocalyx of microglia and oligodendrocytes also contain cell surface sialic acid-binding immunoglobulin-like lectins (SIGLECS), which identify cell surface sialic acid glycoconjugates in adjacent neurons facilitating cellular communication and signalling through an intracellular immunoreceptor tyrosine-based inhibition motif (ITIM); this maintains a homeostatic balance in neuronal cells (Linnartz-Gerlach et al. 2014). Embryonic stem cells assemble a glycocalyx containing cell surface CS epitopes which not only can be used to identify specific stages of stem cell differentiation but serve as interactive modules that respond to instructional cues received from the ECM influencing stem cell differentiation (Furukawa et al. 2016). The glycocalyx displays brain-specific functions through its participation in interactions with cell surface receptors, which undertake protein-phosphorylation-mediated signalling by neurons and can also influence apoptosis and amyloid deposition (Dawson 2014). GAG components in

the glycocalyx have important roles in neuro-protection through interactions with CS-receptors and participation in cell signalling events which maintain cellular integrity and also preserve the tissue hydration provided by GAGs to the pericellular matrix (PCM) and wider ECM. CS-proteoglycans also have regulatory properties in the endothelial and epithelial glycocalyx, which modulate the innate immune response in inflammation and immunomodulation (Chignalia et al. 2016).

4.2.2 GAG Sulphation Motifs as Molecular Recognition and Information Transfer Motifs Which Direct Cellular Behaviour

Sulphation motifs on cell-associated GAGs provide important molecular recognition/activity signals to stem/progenitor cells sequestering growth factors, cytokines and chemokines which ensure stem cell survival in their niche environment (Preston and Sherman 2011), Perturbations in the signals the stem cells receive in this niche can orchestrate stem/progenitor cell differentiation resulting in their activation and proliferation into specific cell lineages and the development of migratory properties and these activated cell lineages can then participate in tissue development and tissue repair. Stem cells in the niche environment are normally maintained in a non-migratory quiescent state where they undergo self-renewal, this is of importance in long-term stem cell survival. Perlecan is highly expressed in the bone marrow and although this PG has cell-binding sites in domains III and IV, it displays anti-adhesive properties in the marrow space towards progenitor/stem cells which serve to compartmentalize them, maintaining these in niches in a slowly recycling state of self-renewal. Fibroblast growth factor-2 (FGF-2) is a major ligand for perlecan, and its sequestration in the niche environment contributes to long-term stem cell viability (Kerever et al. 2007, 2014; Mikedis and Downs 2009; You et al. 2014). HA also regulates bone marrow and nerve stem cell niches by insulating the progenitor cell populations from outside influences and maintaining a cell-friendly environment ensuring long-term survival.

4.3 Stem Cells Are Responsive to Biomechanical Stimuli from the ECM

These cellular events are directed by the composition of the ECM, by biomechanical feed-back cues and GAG sulphation motif interactions with cellular receptors/integrins (Gabius 2015; Gabius et al. 2004; Solis et al. 2001, 2015). GAG chains store and transfer information to cells to provide molecular recognition and activity signals which modulate cell growth and development by regulating growth factors such as the FGF family, *Hedgehog, Wingless*, BMPs, Semaphorins, Midkine and

Pleiotropin (Brickman et al. 1998; Gama et al. 2006; Hacker et al. 2005; Kantor et al. 2004; Palma et al. 2011). Much progress has been made in recent years in our understanding of the contribution of GAGs in tissue development in health and ECM remodelling in disease processes. Until relatively recently, this area of developmental biology had been considered the domain of heparin/HS and the heparanome (Ori et al. 2011; Turnbull et al. 2010). A number of publications on CS however, have now elucidated its specific developmental roles in health and disease (Caterson 2012; Malavaki et al. 2008; Nandini and Sugahara 2006; Purushothaman et al. 2012; Sugahara et al. 2003; Asada et al. 2009). The identification of specific GAG sequences and the charge localizations they provide, their interactive partners, and the biological processes they affect are all areas of importance in tissue development and in repair biology (Gabius 2015; Gabius et al. 2004; Solis et al. 2001, 2015). A greater understanding in this area through basic science studies may improve tissue regeneration strategies and provide the scientific community with new insights into how the pericellular microenvironment surrounding stem/progenitor cell populations regulates cellular senescence and activation, proliferation and differentiation into mature cell populations during tissue growth and development, ECM remodelling and repair processes.

4.4 Phylogenetic Conservation of GAG Structure in Vertebrate and Invertebrate Evolution

GAGs are evolutionarily conserved ancient molecules, which have a widespread distribution in vertebrates and invertebrates but have not so far not been identified in plants (Yamada et al. 2011). The structural diversity of GAGs is clearly evident from many studies (de Paz and Seeberger 2008; DeAngelis et al. 2013; Frey 2015; Rudd et al. 2010) (Fig. 4.1). GAGs are extremely heterogeneous molecules, however, the biosynthesis of complex GAGs such as HS or CS is tightly regulated, and their diverse sulphation patterns occur in an organ and tissue specific as well as in a temporally specific manner during growth and development (Carlsson and Kjellen 2012; DeAngelis 2012; Kreuger and Kjellen 2012; Multhaupt and Couchman 2012; Nadanaka and Kitagawa 2008; Pavao et al. 2006; Zhang 2010). Mutations in the genes which encode the GAG biosynthetic and modification enzymes have serious consequences on the assembly and functional properties of tissues and organs and general well-being, indicating that the expression of specific sulphated recognition structures in GAGs play an essential part in life processes, and this also explains why GAGs have persisted over the millenia (Table 4.1). It is not surprising, therefore, that a significant level of genetic information is invested by cells in the synthesis of complex GAGs such as HS (Fig. 4.2). Biosynthesis of HS requires ~20 enzymes and CS/DS require around 10–12 biosynthetic enzymes, and the recognition aspect of constituent saccharide components in the nascent GAG chain is essential in ensuring the coordination of these complex biosynthetic processes. The aforementioned

Table 4.1 Interactive CS, DS or CS-DS oligosaccharide hybrids [Data modified from (Sugahara 2014)]

Oligosaccharide	Binding partner or property
IB-IB-IB	High-affinity heparin cofactor II binding hexasaccharide
E-E	Tetrasaccharide which stimulates neurite outgrowth activity and also binds to selectins and chemokines
E-E-E-E	Octasaccharide, which binds to Type V collagen
ΔC-C-D-C, ΔA-C-D-C ΔC-A-D-C ΔD-C-D-C ΔC-D-ID-C, ΔE-D-IA-D, ΔA-IB-IB	Pleiotrophin binding octasaccharides isolated from embryonic pig brain
ΔD-C-C-C ΔC-C-A-D	Ovarian cancer octasaccharide epitopes detected by MAb WF6 in serum

information points to the importance of GAGs in essential physiological processes (Table 4.2), which may explain why cells commit such a significant level of genetic material to the conservation of GAG structure throughout vertebrate and invertebrate evolution (Yamada et al. 2011). Given that GAGs are deduced to be essential components in many physiological processes ensuring the survival of the organism, identification of such key life processes is likely to also involve GAGs as active interactive components (Rau et al. 2011).

4.5 CS Sulphation Motifs Identified by Antibodies 4-C-3, 7-D-4 and 3-B-3(−)

Chondroitin sulphate (CS) is a GAG that is composed of β1-3 and β1-4 glycosidically linked D-glucuronic acid and N-acetyl galactosamine *O*-sulphated at the 2, 4 and C6 positions in a repeat disaccharide (Cummings 2009; Caterson 2012). The D-glucuronic acid moiety of CS may be epimerized to αL-Iduronic acid in a related GAG called DS or chondroitin sulphate B. This leads to a considerable degree of structural diversity in CS/DS with over one thousand pentasaccharide sequences possible, which can explore a varied number of interactive structural conformations (Cummings 2009; Caterson 2012). This large array of structures explains why these CS motifs interact with such a diverse repertoire of cytokines, chemokine's, morphogens and growth factors that regulate cellular differentiation and proliferation during tissue development (Cummings 2009; Caterson 2012; Nandini and Sugahara 2006; Sugahara et al. 2003; Pufe et al. 2007). The 3-B-3 (−), 6-C-3, 7-D-4 and 4-C-3 CS sulphation motifs in CS chains are depicted in Fig. 4.1. These were identified in intact native CS chains following partial depolymerization using chondroitinase ABC (Fig. 4.1a, b) (Caterson 2012). Complete enzymatic digestion of the CS

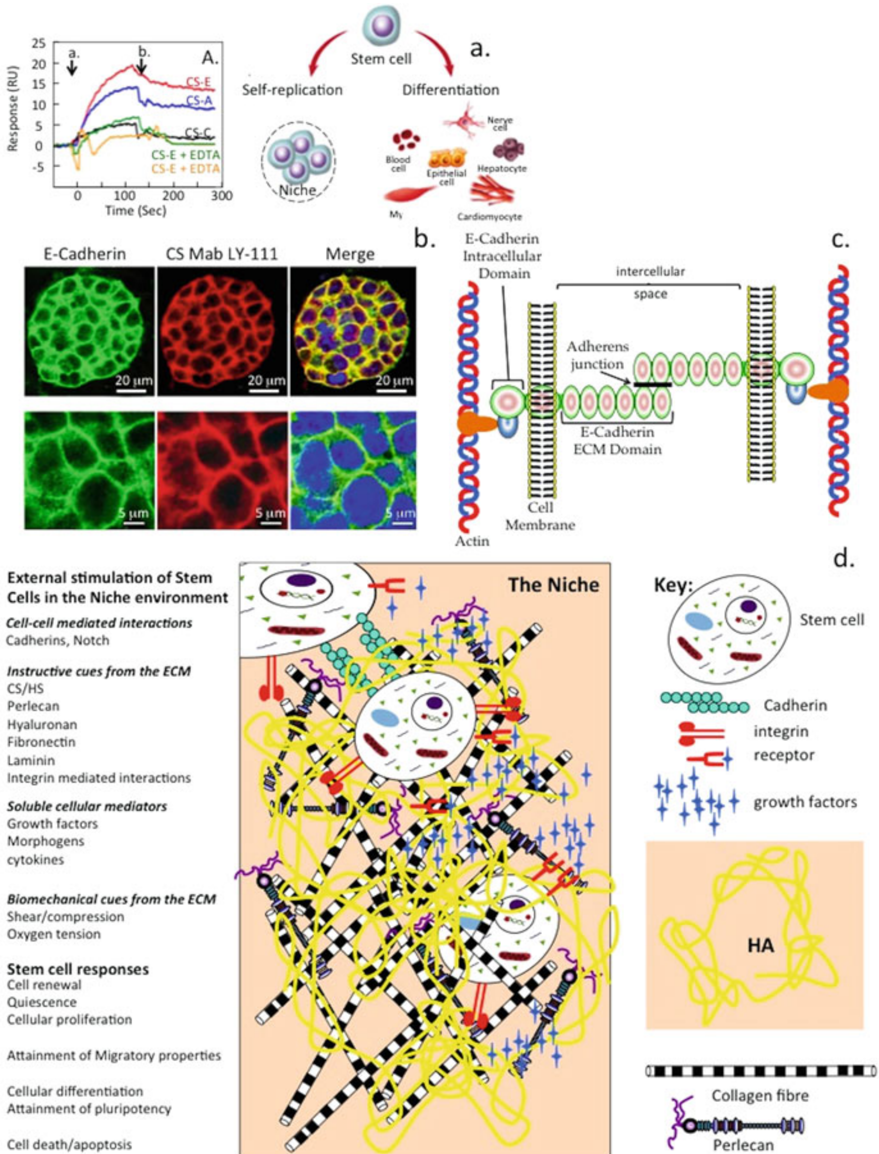

Fig. 4.2 Demonstration of the binding of CS to E-Cadherin using Plasmon resonance (**a**) and colocalization of CS and E-Cadherin by fluorescent microscopy (**b**). CS-E, CS-A or CS-C (400 nM) were injected over recombinant E-Cadherin-Fc immobilized on a biosensor chip in the presence of 3 mM $CaCl_2$ or 3 mM EDTA. The beginning of the association and dissociation phases are marked with arrows **a** and **b**. Cultured Wild type embryonic stem cells stained with antibodies to E-Cadherin and CS (MAb LY111), co-localizations are also shown (**b**). Cell nuclei were stained with DAPI. E-Cadherin and CS colocalize to the adherens junctions between cells. Schematic depiction of the organization of the adherens junction between two adjacent cells which is disrupted by CS (**c**). Figure modified from Izumikawa et al. (2013). Schematic depiction of the stem cell niche and its components and the external stimuli the stem cell experiences and their evoked responses. CS disrupts the E-cadherin bridging shown between adjacent stem cells facilitating stem cell

Table 4.2 GAG disaccharide nomenclature (A) and their structures (B) (Shioiri et al. 2016; Sugahara 2014)

CS disaccharides		DS disaccharides	
Symbol	Sequence	Symbol	Sequence
O Unit	GlcA-GalNAc	IO Unit	IdoA-GalNAc
A Unit	GlcA-GalNAc4S	IA Unit	IdoA-GalNAc4S
B Unit	GlcA2S-GalNAc4S	IB Unit	IdoA2S-GalNAc4S
C Unit	GlcA-GalNAc6S	IC Unit	IdoA-GalNAc6S
D Unit	GlcA2S-GalNAc6S	ID Unit	IdoA2S-GalNAc6S
E Unit	GlcA-GalNAc4S,6S	IE Unit	IdoA-GalNAc4S,6S
K Unit	GlcA3S-GalNAc4S	IK Unit	IdoA3S-GalNAc4S
T Unit	GlcA2S-GalNAc4S, 6S	IT Unit	GlcA2S-GalNAc4S, 6S

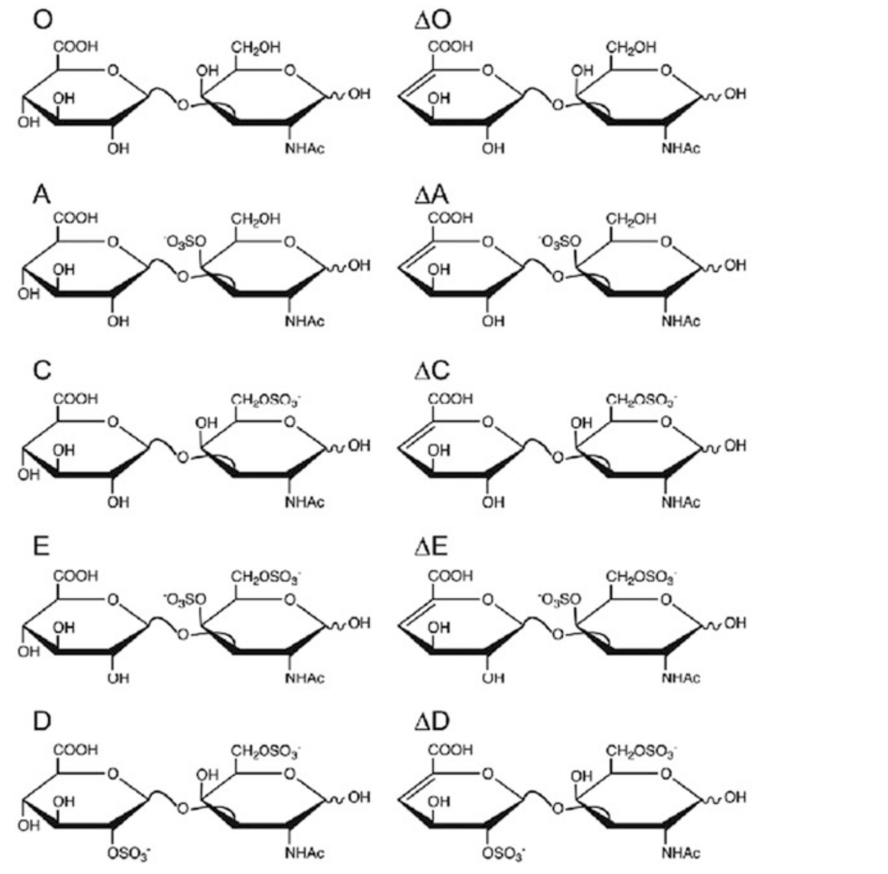

Fig. 4.2 (continued) differentiation and attainment of pluripotency (**d**). Only one proteoglycan is shown for clarity. Perlecan is a CS-HS hybrid in bone marrow, cartilaginous and myogenic niches

chain using chondroitinase ABC generates unsaturated 3-B-3 (+) and 2-B-6 (+) stub disaccharide epitopes (Fig. 4.1c). These should not be confused with the 3-B-3 (−) and 2-B-6 (−) epitopes, which are terminal epitopes generated by HYAL 4 (Fig. 4.1d).

The mast cell PGs serglycin and perlecan have been reported to display a 2-B-6 (−) epitope on their CS chains (Farrugia et al. 2016a). The 3-B-3 (−), 2-B-6 (−) epitopes are not generated by chondroitinase ABC digestion. The 2-B-6 (−) epitope is present in osteoarthritic cartilage (Asari et al. 1996) and is generated by the action of HYAL-1 or HYAL-4, CS hydrolases which cleave CS in the β1→4 disaccharide glycosidic linkage (Yamada et al. 2009). The 2-B-6 (−) and 3-B-3 (−) epitopes produced by HYAL-4 occur in mast cells and are associated with tissue development and with connective tissue remodelling in inflammatory conditions (Farrugia 2019, #269). Chondroprogenitor cells may also express HYAL-4 and generate the 3-B-3 (−) epitope attached to a different PG to that found in mast cells.

4.5.1 Identification of the CS Hydrolase: "the Mammalian Chondroitinase" as the Generator of the 3-B-3(−) and 2-B-6(−) CS Sulphation Motifs

With regard to the turnover of GAG components of connective tissues, there has long been conjecture as to how this is achieved with CS in the absence of a known mammalian chondroitinase. Since CS is the most abundant GAG in the human body, this was a major unexplained anomaly. Specific hydrolases exist for the turnover of HA and HS and sulphate editing enzymes (Sulf 1, 2, aryl sulphatase) can also modify their sulphation motifs (Schartz et al. 1974; Shikhman et al. 2000). Some of the hyaluronidases can degrade CS but only to a limited extent. Furthermore, eight GAG degrading glycosidases and glycoside sulphatases are produced by articular chondrocytes, including hexosaminidase, hexosaminidase A, N-acetyl-α-D-glucosaminidase, β-galactosidase, β-glucuronidase, α-L-iduronidase, aryl sulfatase, galactose-6-sulphate sulfatase (Yamada 2015). IL-1 selectively upregulates hexosaminidase and β-galactosidase production by chondrocytes, and these are active at neutral pH in the ECM. Even with the aforementioned glycosidases, chondrocytes are ill-equipped to turn over the major CS component of the ECM. The recent discovery that Hyal4 was actually a CS hydrolase rather than a hyaluronidase was therefore interesting. Hyal4 and Hyal 1 generate non-reducing terminal 3-B-3(−) and 2-B-6(−) epitopes, however, the significance of these epitopes in connective tissue pathobiology has yet to be fully determined (Farrugia et al. 2016b, 2019). Numerous studies document that the 3-B-3(−) and 2-B-6(−) epitopes are associated with tissue development and appear to decorate cell surface PGs synthesized by activated stem cell populations in transitional tissues undergoing morphogenetic changes in tissue development. GAGs have binding properties for a range of growth factors, chemokines and cytokines in tissues and act as cellular mediators in cell

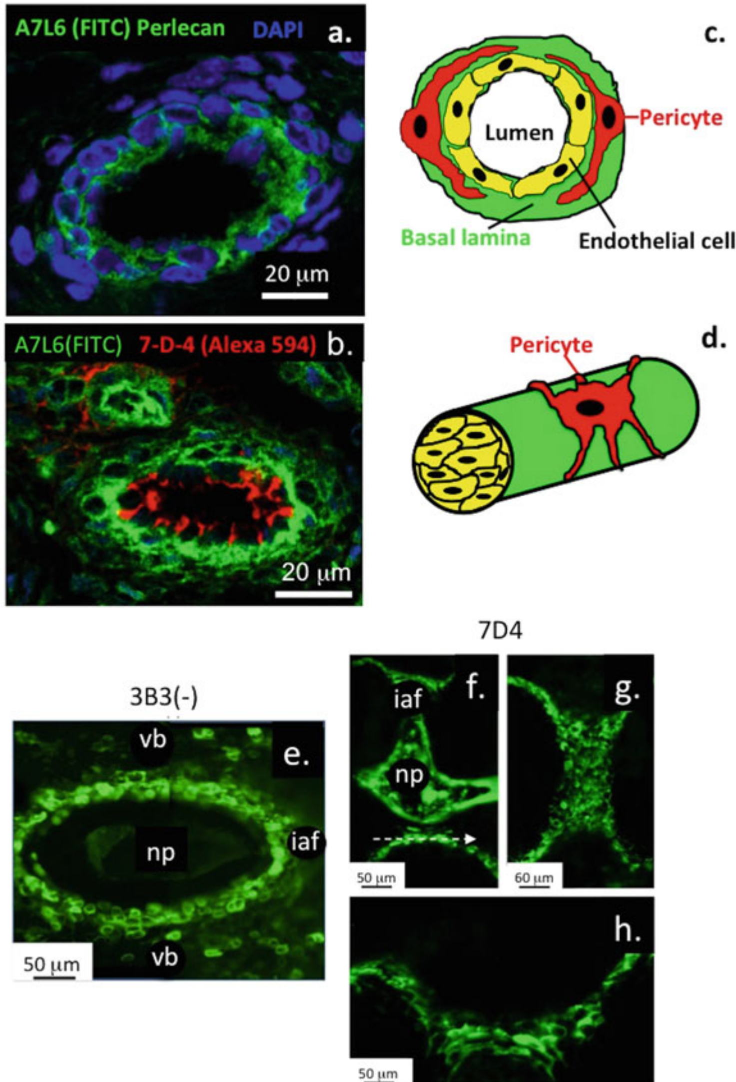

Fig. 4.3 Putative vascular stem cell niche depicting the focal expression of perlecan (**a**) and the 7-D-4 CS sulphation motif (**b**) by endothelial cells in the lumen and communication between pericytes associated with the vessel wall and endothelial cells (**c**, **d**). The confocal images shown are from human foetal paraspinal blood vessels (14 weeks gestational age)

signalling, however, the specific ligand binding properties of the 3-B-3(−) and 2-B-6(−) epitopes are yet to be determined. Immunolocalization of the 3-B-3(−) epitopes in foetal human and rat IVD tissues (Fig. 4.3e–h) show these have a very specific spatiotemporal cellular distribution in tissue development associated with progenitor cell populations and stem cell niches in the rudiment cartilages during

diarthrodial joint and in the transitional zone in intervertebral disc development (Hayes et al. 2011, 2016, 2018b) (Fig. 4.3e–h). The 3-B-3(−) epitope has also been identified in bone development thus may be markers of osteoprogenitor cells as well as chondroprogenitor and discoprogenitor cell populations (Li et al. 2010) (Figs. 4.4, 4.5, 4.6). Furthermore, a recent in vitro study with bone marrow-derived mesenchymal stromal stem cells showed that FGF-18 could induce CS sulphation motif expression (4-C-3, 7-D-4) in micro-mass pellet culture which co-localized with areas of calcium deposition (Shu et al. 2016). Osteogenic gene expression was also promoted by FGF-18, which is consistent with the strong immunolocalization of FGF-18 at the cartilage-bone interface (Fig. 4.7e–g) and in the long bone growth plates (Fig. 4.7a–e) while FGF-2 was localized to the chondrocytes within the articular cartilages (Fig. 4.8a–f) (Shu et al. 2016).

It will be interesting in future studies to determine if the 3-B-3(−) and 2-B-6(−)(−) epitopes have specific binding properties for some key molecules with roles in tissue development or in the turnover of connective tissues in health and disease. At present, the 3-B-3(−) CS sulphation motif is a useful biomarker of activated stem cell populations with roles in tissue development. It will be interesting to fully determine the specific roles of these CS sulphation motifs in stem cell maturation, tissue development and homeostasis.

Human hyaluronidase-4 (HYAL4) is a CS-specific endo-beta-*N*-acetyl-galactosaminidase that has been isolated from *Caenorhabditis elegans* (Yamada et al. 2009*;* Kaneiwa et al. 2008*)* and is also expressed in a few mammalian tissues. HYAL4 degrades chondroitin, chondroitin-4-sulphate and chondroitin-6-sulphate, however, HA is a relatively poor substrate for this enzyme; thus HYAL4 should be more appropriately considered as a chondroitin hydrolase and is the only animal enzyme so far identified with this enzymatic activity. HYAL4 cleaves the galactosaminidic linkage in a trisulphated tetrasaccharide sequence in CS in aggrecan and recombinant serglycin (Honda et al. 2012; Kaneiwa et al. 2010, 2012). Expression of HYAL4 mRNA is not ubiquitous but has so far been demonstrated to be restricted to the placenta, skeletal muscle, and testis (Csoka et al. 1999, 2001), indicating that HYAL4 is apparently not responsible for the systemic catabolism of CS observed in pathological cartilaginous tissues, and the generation of the 2-B-6(−) epitope in OA cartilage (Asari et al. 1996). We hypothesize that HYAL4 may be responsible for the production of 3-B-3(−) and 2-B-6(−) in inflammatory pathologies, due to the infiltration of mast cells, and this could be why these epitopes are also seen in fibrillated OA cartilage where the mast and other immune cells are also likely to be present. We also hypothesize that Hyal1/4 may also be produced by other inflammatory cells. Further enzymes related to HYAL4 with different tissue distributions may therefore await discovery (Jedrzejas and Stern 2005; Yamada 2012). The recent development of a PG-substrate gel zymographic electrophoretic technique should facilitate the detection of such enzymes (Kobayashi et al. 2018). HYAL4 has recently been detected in a rat spinal cord hemi-section model (Tachi et al. 2015) where CS-PGs are upregulated around the spinal defect site from 4 days after cordotomy and remain increased until 5-6 weeks after injury. HYAL-4 was also immunolocalized around the defect site from 4 days post-injury and identified in

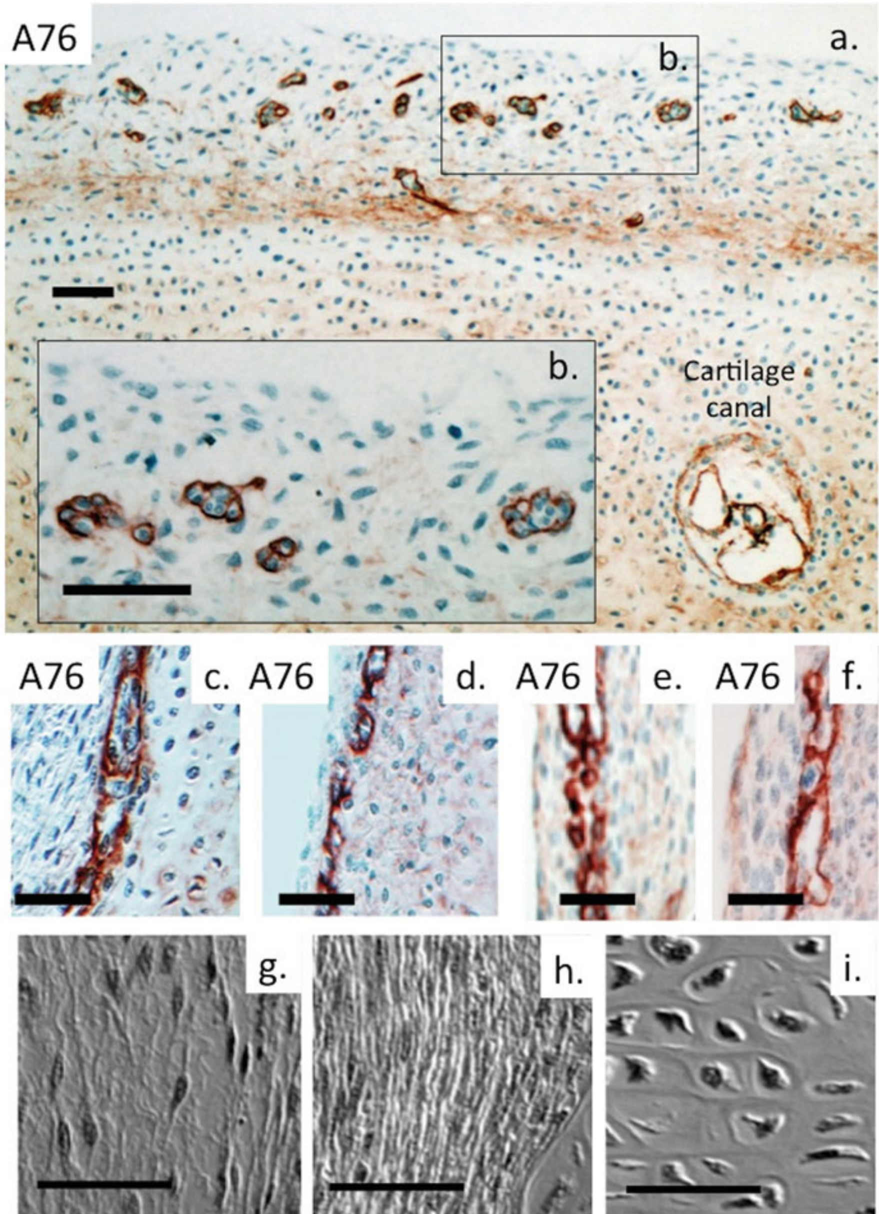

Fig. 4.4 Perlecan expression in human foetal (14 week gestational age) hip (**a**, **b**) and knee cartilage rudiments (**c**–**f**) delineating isolated stem cell niches in the surface of the cartilage rudiments. The morphological characteristics of stromal cells associated with a knee rudiment (**g**), surface zone rudiment cells (**h**) and chondrocytes within the rudiment (**i**) in unstained sections using Nomarski differential interference contrast microscopy. Perlecan was immunolocalized using anti-perlecan domain IV MAb A7L6. The inset (**b**) shows the selected boxed region in (**a**) depicted at higher magnification to visualize stem cell niches

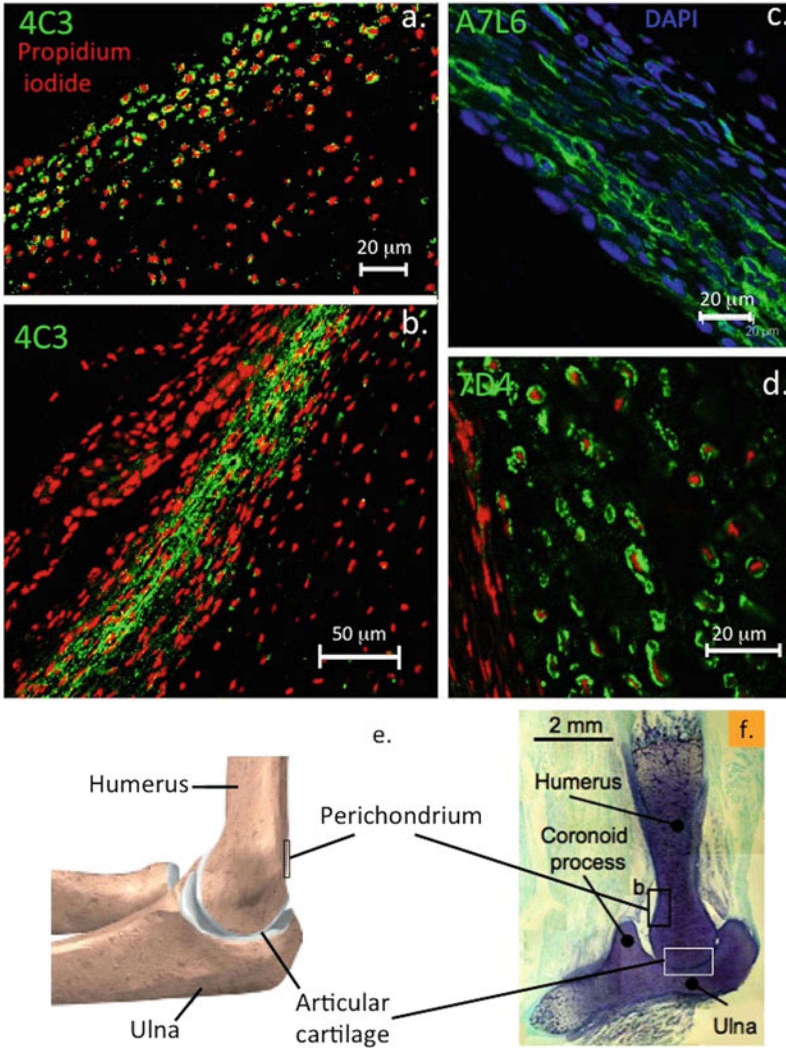

Fig. 4.5 Stem cells expressing the 4-C-3 CS sulphation motif at the articular surface of the radius (**a**) and by progenitor cells also expressing perlecan in the perichondrium (**b**, **c**, **d**) of a human foetal elbow (14 weeks gestational age)

tissue extracts by Western blotting. Double staining for CS and HYAL4 confirmed the colocalization of CS56 and HYAL4 around the spinal cord defect (Tachi et al. 2015). Thus HYAL4 displayed a spatiotemporal localization pattern promoting tissue remodelling and spinal cord repair similar to that seen for 3-B-3(−) in the development of connective tissues during tissue morphogenesis. Furthermore, the use of an antibody to HYAL4 prevented remodelling of the spinal cord defect site (Yokoyama et al. 2019), demonstrating a role for HYAL4 in the remodelling of

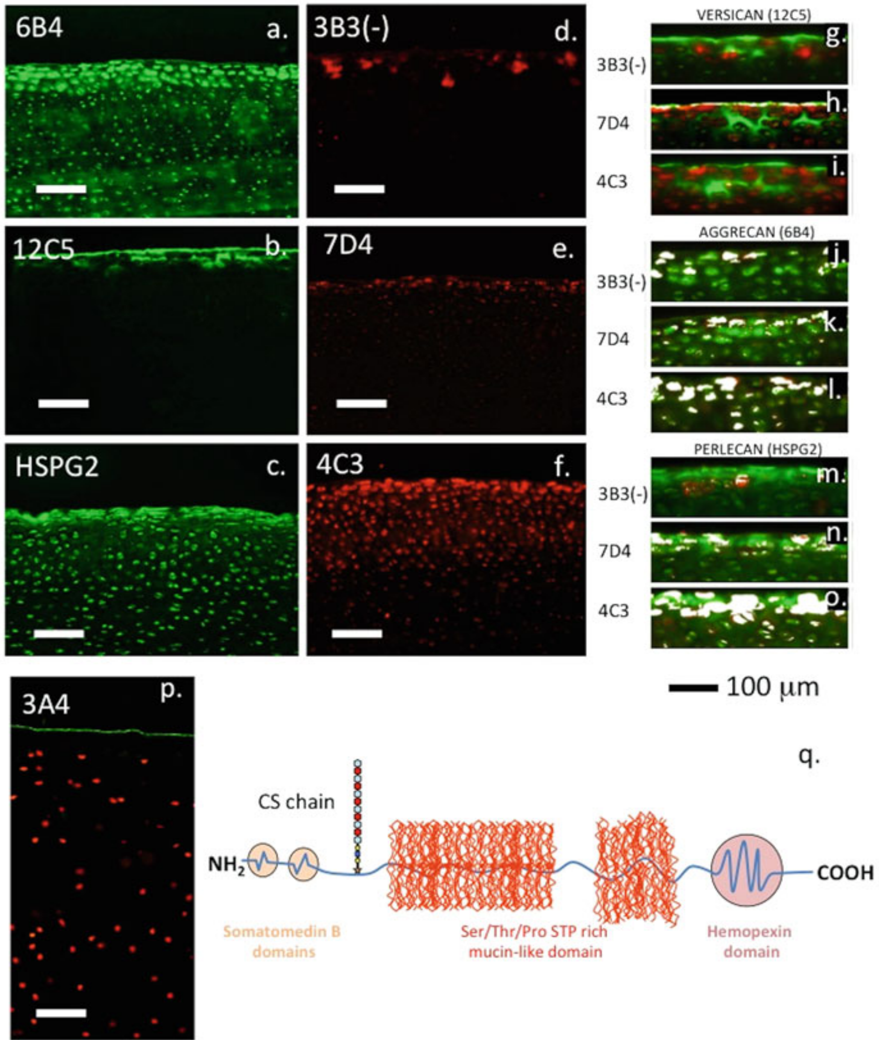

Fig. 4.6 Immunolocalization of aggrecan (6B4, aggrecan IGD) (**a**), versican (12C5, versican G1 domain) and perlecan (HSPG2) (**c**) and co-expression of 3-B-3(−) (**d**), 7-D-4 (**e**) and 4-C-3 (**f**) CS sulphation motifs in confocal vertical sections of 1-year-old bovine articular cartilage Co-localization of the 3-B-3(−), 7-D-4 and 4-C-3 CS sulphation motifs with aggrecan (**a–c**), versican (**d–f**) and perlecan immunolocalizations (**g–i**) in fluorescent immunolocalizations of bovine articular cartilage. Areas of co-localization are shown using a white voxel overlay (**j–o**). Versican only co-localizes with MAb 7-D-4 in the surface most regions of the articular cartilage while aggrecan and perlecan are more widely distributed and co-localize strongly with MAb 4-C-3. The 3-B-3(−) CS sulphation motif identifies small discrete groups of cells in the surface region of the articular cartilage consistent with the presence of small stem cell niches in this region. Lubricin (PRG4) is also a prominent component at the articular surface (**p**, **q**)

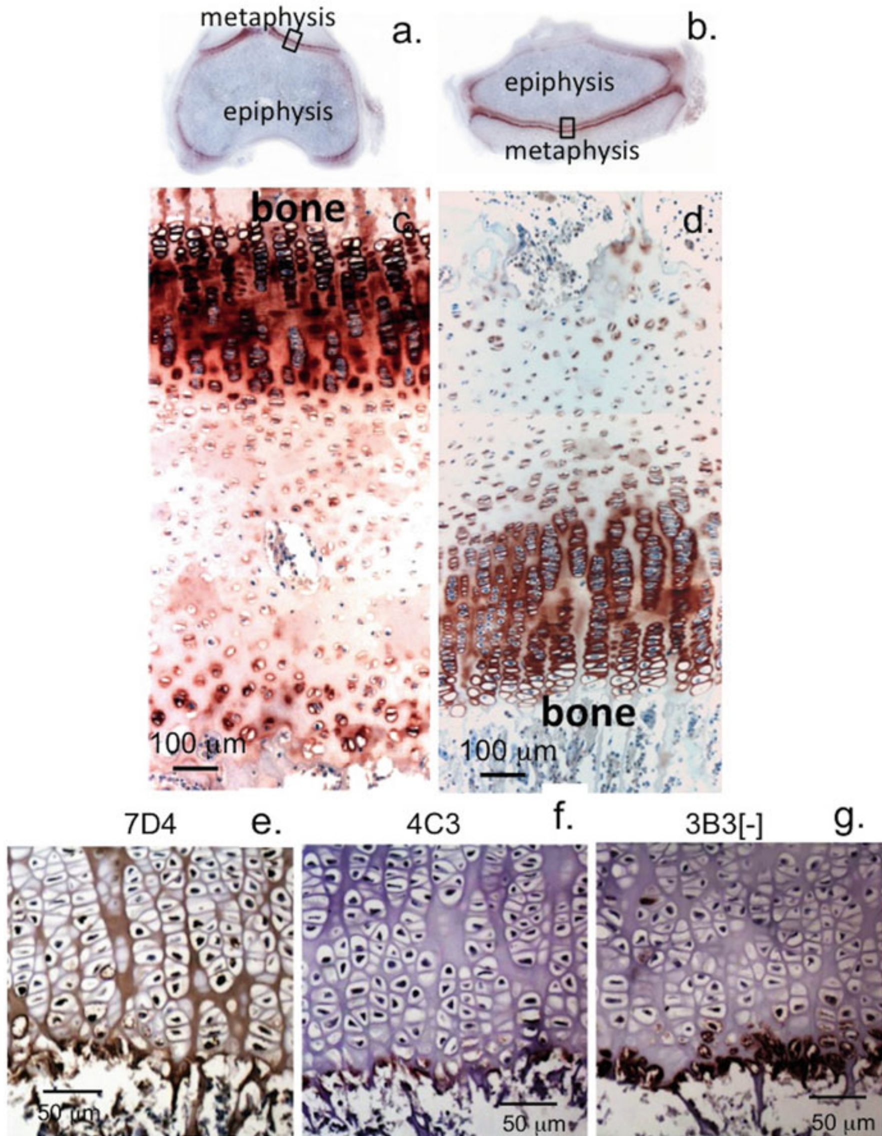

Fig. 4.7 Immunolocalization of FGF-18 in the young ovine knee and hip cartilages (**a**–**d**) and immunolocalization of the CS sulphation motifs 7-D-4, 4-C-3, and 3B3 (+) in human foetal tibial growth plate cartilage (**e**–**g**)

CS-PGs which accumulate in the cord defect site (Tachi et al. 2015). The 3-B-3(−) CS sulphation motif is a prominent component of the surface articular cartilage of the cartilage rudiments during foetal human diarthrodial joint development (Fig. 4.9a, b). The 7-D-4 CS sulphation motif is also expressed in this surface region

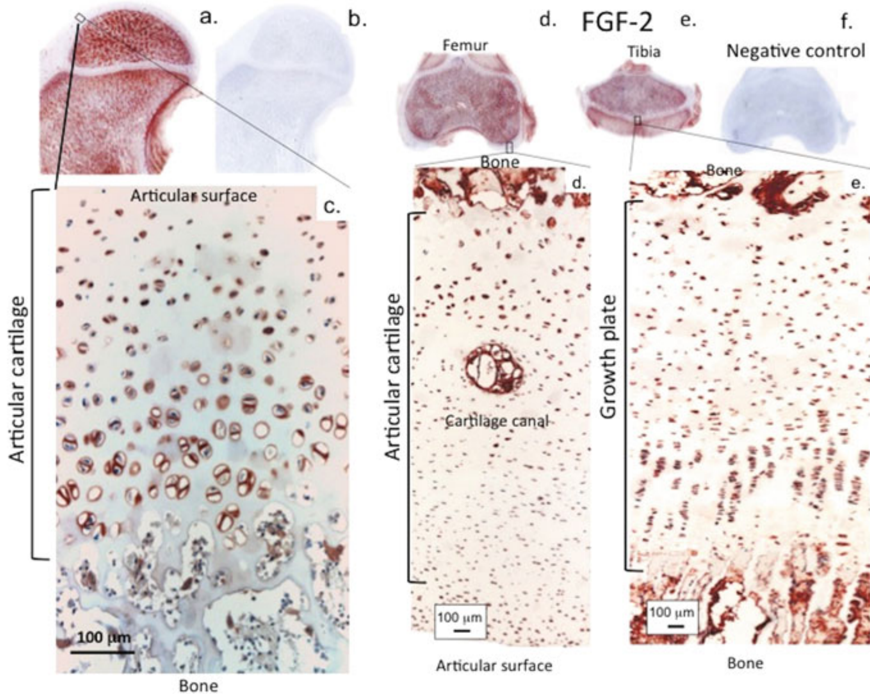

Fig. 4.8 Immunolocalization of FGF2 in the postnatal ovine knee and hip cartilages

of rudiment articular cartilage however, the distribution of this component is confined to a discrete collection of small groups of cells and has a pericellular distribution (Fig. 4.9c, d). This contrasts with the 3-B-3 (−) epitope which has an extensive extracellular distribution around cells in the surface regions of the rudiment cartilage of the developing diarthrodial joint and is consistent with the 3-B-3(−) epitope being located on aggrecan which is a prominent major component of the rudiment cartilage. The 3-B-3 (−) epitope can be generated in vitro on the CS chains of aggrecan through the action of HYAL4 (Farrugia et al. 2019). In vitro digestions with HYAL4 also generated the 3-B-3(−) epitope on Serglycin (Fig. 4.9f, h), which is known to be produced by embryonic stem cells (Schick et al. 2003). HYAL4 also has previously unidentified roles in cartilage rudiment remodelling during diarthrodial joint development. This is consistent with roles for HYAL4 spinal cord remodelling in a hemisection model of spinal cord injury (Tachi et al. 2015; Yokoyama et al. 2019). Concomitant with the generation of the 3-B-3(−) epitope by HYAL4, there is also a reduction in CS56 epitope reactivity (Fig. 4.9e, g), confirming the action of HYAL4 on the CS chains (Farrugia et al. 2019). The epitope for CS56 is an extended octasaccharide sequence containing CSA-D tetrasaccharides (Deepa et al. 2007) thus HYAL4 must cleave this epitope however we also deduce that the cleaved CS chain remaining attached to proteoglycan core protein is terminated by a non-reducing terminal 3-B-3 epitope.

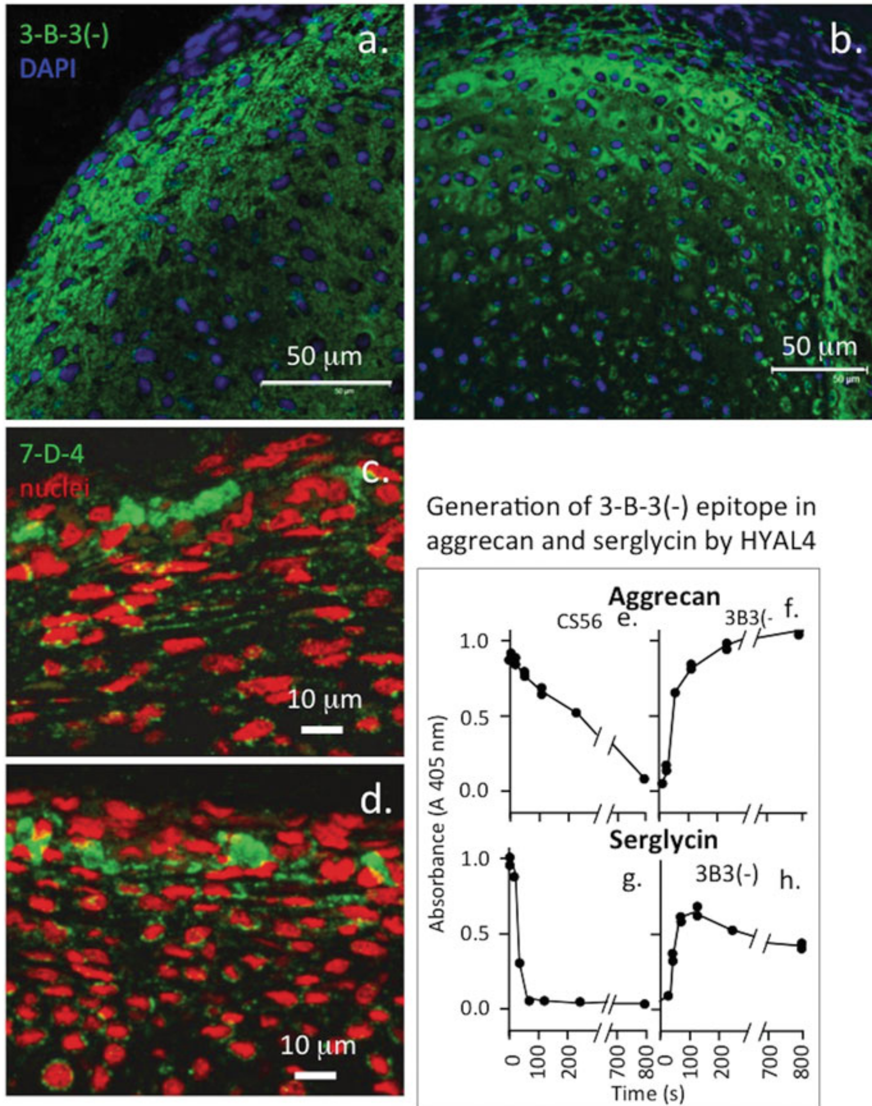

Fig. 4.9 Immunolocalization of the 3-B-3 (−) (**a**, **b**) and 7-D-4 CS sulphation motifs (**c**, **d**) in human foetal (12 weeks gestational age) knee joint development and demonstration of the 4-B-3(−) CS epitope by digestion of the CS chains of aggrecan and serglycin by HYAL4 (**f**, **h**). Segments (**f**, **h**) reproduced from Brooke L. Farrugia, Shuji Mizumoto, Megan S. Lord, Robert L. O'Grady, Rhiannon P. Kuchel, Shuhei Yamada and John M. Whitelock. Hyaluronidase-4 is produced by mast cells and can cleave serglycin chondroitin sulphate chains into lower molecular weight forms. J. Biol. Chem. jbc.RA119.008647. https://doi.org/10.1074/jbc.RA119.008647 with permission © the Author(s). under the auspices of CC-BY licence

4.6 GAGs and Tissue Repair: GAG Bioscaffolds Which Promote Stem Cell Differentiation

Two decades ago, GAGs were chemically grafted to type I collagen for use as a scaffolding material to promote skin repair and peripheral nerve regeneration (Ellis and Yannas 1996). GAG bioscaffolds have been demonstrated as promising tissue repair agents (Schnabelrauch et al. 2013). CS is the major GAG in the human body and is assembled from a number of isomeric CS disaccharides of variable sulphation, which provides a polymer with a biodiverse repertoire of ligand interactivity. Practical considerations dictate that readily available and inexpensive GAG sources are used in the preparation of bioscaffolds. An appreciation of the biological heterogeneity of naturally occurring GAGs is mandatory if they are to be optimally utilized in routine applications and reproducible therapeutic responses obtained through standardized therapeutic protocols. Non-mammalian and synthetic GAG sources are also available (Pomin 2015). The synthesis of CS and DS homopolymers with accurately controlled repeat disaccharides produce scaffolds of well-defined reproducible physical attributes, also an important consideration when considering how the material properties of such scaffolds can influence the differentiation of therapeutic cell populations seeded into or migrating into the scaffold in situ.

It should be appreciated that the biological properties of specific GAG motifs embedded within the main GAG chain may be a major biological determinant in some PGs, thus micro-sequence information on the GAG chains is very useful information to have, however unfortunately, sequencing of small GAG samples is normally beyond the capability of most laboratories. The CS-A chains of some PGs may contain variable levels of embedded CS-D or CS-E disaccharide motifs, the high charge density provided by di-sulphated CS-D and CS-E motifs convey important cell signalling properties which can promote collagen fibrillogenesis (Kvist et al. 2006), chondrogenesis, and nerve re-growth (Hayes and Melrose 2018).

Methods have been developed to synthesize CS tetrasaccharides containing 4,6-disulphated residues and their protein binding profiles determined, but they have yet to be systematically employed in repair biology (Miyachi et al. 2015). The CS-A chains of bikunin have embedded CS-D motifs which convey interactive properties to their CS chains with HA (Lord et al. 2013). TSG-6 catalyzes the transfer of ITI and pre-α-ITI HC chains to HA in tissues such as the growth plate, lung and reproductive tissues (Milner et al. 2007). This cross-links and stabilizes the HA and is of importance in fertilization, oocyte metamorphosis and stabilization of tissues. The brain mouse CS-PG, DSD-1-PG/6B4-PG/phosphacan displays neurite outgrowth-promoting activity attributable to the disulphated DSD-1 disaccharide unit GlcUA(2S)-GalNAc(6S) embedded in its CS side chains, however, the CS-A disaccharide which constitutes the majority of the GAG chain inhibits neural outgrowth (Hikino et al. 2003). Oversulphated CS-D from shark cartilage and CS-E from squid cartilage possess similar neural outgrowth properties. A marine sea cucumber CS trisaccharide containing a sulphated fucose branch point also exhibits neurite outgrowth-promoting activity (Shida et al. 2017). A number of

neuritogenic DS GAGs have been identified in marine organisms (Valcarcel et al. 2017). CS-E is the only GAG in free solution which can modulate collagen fibrillogenesis in vitro, promoting the formation of thin collagen fibrils rather than thick collagen fibres such as those which occur during tissue fibrosis. Oversulphated GAGs have been incorporated into bioscaffolds to promote the differentiation of osteoblast-precursor cells and premature osteoblasts. The aforementioned oversulphated GAGs from marine organisms may also be applicable in such studies on the differentiation of osteoblast progenitors but have yet to be evaluated for this purpose.

The CS chains of the brain PG Appican contain embedded CS-E domains which also promote neuritogenesis. CS hydrogels have been developed in an attempt to recreate an environment conducive to the formation of a stem cell niche (Karumbaiah et al. 2015) to promote adipose-derived stem cell differentiation and improved dermal repair (Matsumine et al. 2017). The ability of GAG hydrogel wound dressings to sequester inflammatory chemokines such as MCP-1 (monocyte chemoattractant protein-1), IL-8, MIP (macrophage inflammatory protein)-1α/β reduces the bioavailability of these chemokines in chronic wound fluids, improving the healing of leg ulcers (Lohmann et al. 2017). Gelatin-CS nanofibrous scaffolds also sequester therapeutic growth factors which stimulate wound healing (Pezeshki-Modaress et al. 2016) and have been explored as an IGF-I delivery system to promote cartilage repair (Mullen et al. 2015) and when supplemented with FGF-18 and BMP-7 have been used in osteochondral repair (Getgood et al. 2014). Highly sulphated GAG-collagen scaffolds are anti-inflammatory preventing the secretion of IL-6, IL-8, MCP-1, and prostaglandin E2 in response to stimulation with IL-1β and promote osteogenic differentiation of stem cells (Hempel et al. 2014a, b).

4.6.1 Prospective Roles for Biglycan, Decorin and FGF-18 in Osteogenesis

4.6.1.1 Biglycan and Decorin, Modulate Bone Marrow Stromal Cell Differentiation, Co-ordinate TGF-β Sequestration in the ECM and Regulate Bone Deposition

Decorin and biglycan modulate the proliferation and survival of bone marrow stromal cells (Bi et al. 2005). In the absence of these proteoglycans, TGF-β binds directly to receptors on the bone marrow stromal stem cells over-activating their signal transduction pathways resulting in a switch from growth to apoptotic signalling, a reduction in osteoprogenitor cell numbers and reduced bone growth. Biglycan and decorin are essential for the maintenance of mature osteoblast numbers by modulating the proliferation and survival of bone marrow stromal cells. Lentiviral delivery of biglycan promotes proliferation and increases the osteogenic potential of bone marrow-derived mesenchymal stem cells in vitro (Wu et al. 2013). TGF-β

promotes the expression of biglycan, decorin and alkaline phosphatase in osteoblast-precursor cells which maintains differentiated osteoblast numbers (Yamada et al. 1999). Biglycan mediates suture expansion by promoting osteogenesis (Wang et al. 2015) through BMP mediated interactions (Miguez et al. 2014), and promotes angiogenesis during bone formation and fracture healing (Berendsen et al. 2014). FGF-18 is required for normal chondrogenesis (Davidson et al. 2005) and osteogenesis (Haque et al. 2007; Ohbayashi et al. 2002), and co-ordinates these processes (Liu et al. 2002), promotes early chondrogenesis and cellular maturation leading to osteogenesis of cultured bone marrow stromal cells (Shu et al. 2016; Jeon et al. 2012), regulating the vascular invasion of the growth plates in vivo (Liu et al. 2007) and accelerating osteoblast differentiation through an up-regulation in BMP-2 expression (Nagayama et al. 2013).

4.7 FGF-18 Induces Osteogenic Differentiation of Chondrocytes and CS Sulfation Motif Expression by Osteoprogenitor Stem Cells In Vitro

In micro-mass pellet cultures of bone marrow-derived stromal stem cells, FGF-18 promoted early proliferation and chondrogenesis followed by osteogenesis of chondroprogenitors selected from the total bone marrow stromal stem cell pool (Shu et al. 2016). FGF-18 promoted cell proliferation and chondrogenesis on days 21–31 compared to basally cultured bone marrow stromal cells in chondroselection media only. Expression of osteogenic genes (Mef2c, Alpl) was accompanied by a down-regulation in Acan, and Col2A1 on day 41. Deposition of Calcium in the central region of the pellet (Alizarin stain) was accompanied by the expression of the CS sulphation motifs 4-C-3 and 7-D-4, which did not occur in basal or FGF-2 stimulated pellet cultures. Focal expression of FGF-18 by hypertrophic chondrocytes in the growth plate cartilages of the knee and hip joints is consistent with FGF-18 promoting bone formation at the chondro-osseous junction. FGF-18 also stimulated decorin and biglycan production by the osteoprogenitors in the pellet cultures. These PGs were also immunolocalized in the central region of pellets where calcium deposition and expression of the 4-C-3 and 7-D-4 CS sulphation motifs were observed, suggesting decorin or biglycan may be decorated with the 4-C-3 and/or 7-D-4 motifs. These motifs are also expressed by progenitor cells in stem cell niches in articular cartilage surface regions and growth plate chondrocytes at the cartilage bone-interface during diarthrodial joint development. The current observations indicate that these CS sulfation motifs apparently represent bio-markers of osteoprogenitor cells at the cartilage-bone interface and may be carried by decorin or biglycan synthesized by the osteoprogenitor cells.

4.7.1 FGF-18 Induces Expression of Specific CS Sulfation Motifs by Osteoprogenitor Cells

Due to their small sizes, it was not possible to separately isolate total RNA from the inner and outer regions of the pellets despite the obvious differences in the immunolocalizations conducted on these regions. This may explain the wide spread in qRTPCR data presented in Fig. 4.10b–d. This needs to be borne in mind when interpreting this data; thus the high immunolocalization of decorin and biglycan in the inner core region of the pellet is not represented as strongly as expected by the gene expression data, even so, this data still showed elevated expression levels of decorin and biglycan. Likewise, the reduction in perlecan expression in FGF-18 RTPCR data would have been less significant if the outer pellet region had been sampled in isolation.

Endochondral bone formation is an intricate process that begins with the differentiation of mesenchymal cells to chondroblastic cells to form mesenchymal condensations. The chondroblasts proliferate, differentiate and lay down ECM to expand the mesenchymal cell mass, eventually exiting from the cell cycle to reach terminal differentiation. This leads to the appearance of hypertrophic chondrocytes, which undergo programmed cell death and are replaced by osteoblasts, which undertake the transition of cartilage to bone (Karsenty and Wagner 2002). Signalling molecules such as Indian hedgehog (Ihh), parathyroid hormone-related protein (PTHrP), fibroblast growth factors (FGFs), Wnt and BMPs, coordinately regulate this. Ihh signalling regulates chondrocyte proliferation and differentiation (St-Jacques et al. 1999) but delays the progression of chondrocytes to a hypertrophic state by the induction of PTHrP, which signals proliferative chondrocytes and prevents them from becoming hypertrophic (Lanske et al. 1996). Ihh regulates chondrocyte proliferation independently from PTHrP to control the rate of cell division by the columnar proliferative chondrocytes (Long et al. 2001) and also regulates the progression of the resting chondrocytes to a proliferative state in the growth plate (Kobayashi et al. 2005). Cbfβ has a key role to play in endochondral ossification by coordinating the up-regulation of Ihh and inhibition of PTHrP receptor expression to control the balance between chondrocyte proliferation and differentiation in postnatal cartilage (Tian et al. 2014). The sulfation status of ECM CS-PGs is also important in Ihh signalling in the developing growth plate (Cortes et al. 2009). Undersulfation of CS-PGs in the brachymorphic mouse results in reduced Ihh signalling and abnormal Hh protein distribution and skeletal defects due to altered endochondral ossification (Cortes et al. 2009). Ihh binds to CS-PGs, specifically aggrecan, the most abundant cartilage PG together with HS-PGs modulate Ihh signalling by controlling the ECM distribution of secreted Ihh. This interaction of Ihh with CS-PGs and the modulation of Hh signalling demonstrates the importance of the sulfation status of CS and how Ihh can act as a long-range morphogen in skeletal development.

Chondroitin 4-O-sulfation by *C4st1* is required for proper CS localization, modulation of distinct signalling pathways and cartilage growth plate morphogenesis (Kluppel et al. 2005). Mutation in the chondroitin-4 sulfotransferase-1 gene (*C4st1*

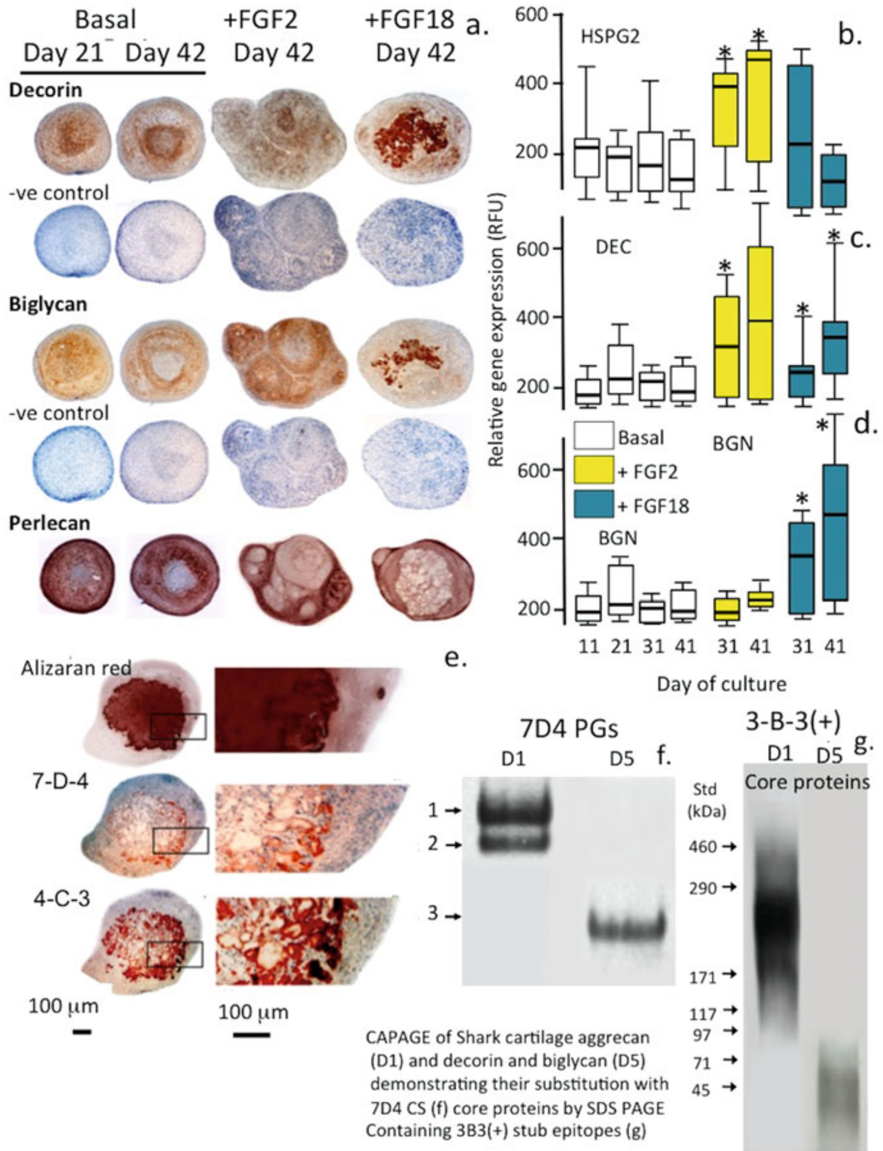

Fig. 4.10 Immunolocalization of decorin, biglycan and perlecan expression by bone marrow stromal Mesenchymal cells (MSCs) grown in micro-mass pellet cultures stimulated with FGF-2 and FGF-18 (**a**) and qRTPCR gene profiling (**b**). The CS sulphation motifs 7-D-4 and 4-C-3 are also produced by MSCs cultured in the presence of FGF-18 and osteogenic genes are upregulated. Calcium deposition in chondrocyte pellets depicted by Alizarin red staining has a similar distribution to the 7-D-4 and 4-C-3 localizations (**e**). Isolation of aggrecan and decorin/biglycan in GuHCl extracts of shark cartilage by isopycnic CsCl density gradient ultracentrifugation in the D1 and D5 fractions, respectively, demonstrated they were of widely differing buoyant densities and were substituted with the 7-D-4 sulphation motif (**f**) and 3B3 CS chains (**g**)

gt/gt) causes chondrodysplastic disorganized cartilage growth plates, alterations in the orientation and spacing of chondrocyte columns and an imbalance in apoptotic signals in the growth plate. *C4st1 gt/gt* mutation leads to strong up-regulation of TGF-β signalling, down-regulation of BMP signalling, while Ihh signalling was unaffected (Kluppel et al. 2005).

4.7.2 Modulation of Stem Cell Activity by Extrinsic Forces

In addition to biochemical signals which regulate stem cells in situ, mechanical cues also play regulatory roles in stem cell differentiation in the niche environment. Stem cells are responsive to extrinsic mechanical forces, and these are important determinants of stem cell differentiation, fate and lineage commitment. Stem cells contain mechanical sensors which equip them to perceive and respond to mechanical signals. The nucleus is the largest and stiffest cellular organelle and its interaction with cytoskeletal proteins has essential roles in the mediation of cellular mechanics. Nuclear mechanics involve sophisticated interactions between lamins, chromatin, nucleoskeletal-proteins and transcription factors through which stem cell differentiation is undertaken in response to mechanotransduction and mechanical cues from the ECM (Du et al. 2016; Mao et al. 2015).

Transmission of mechanical cues (compression/shear) from the ECM can modulate stem cell activity, ECM geometry/topography and substrate rigidity at the nanoscale level all contribute to how stem cell behaviour is affected by their local microenvironment (Guilak et al. 2009). Matrix-mediated signals include mechanical stimuli such as strain, shear stress, forces transmitted through substrate rigidity and variable matrix topography (Delaine-Smith and Reilly 2012; Lv et al. 2015; Saldana et al. 2014). The mechanome of live stem cells has been mapped by measuring local strain fields at the fluid-cell interface (Song et al. 2012).

Self-renewal and lineage commitment of stem cells are directed by mechanical cues through integrin-mediated focal adhesions, which anchor the stem cells in the ECM, allowing it to sense the surrounding microenvironment and to react to changes in the ECM (Subramony et al. 2013; Wang and Chen 2013). Substrate-cell and cell-cell interactions both activate specific mechano-transductive pathways that regulate stem cell fate (Nava et al. 2012). Feed-on mechanical cues to the stem cell are influenced by substrate stiffness, surface nano-topography, micro-geometry and ECM compressive and shear forces (Yim and Sheetz 2012). Dynamic pressurization of notochordal cells within the NP induces their transition to a mature phenotype (Purmessur et al. 2013), compression (hydrostatic or osmotic pressure) or a combination of shear and dynamic compression, also induces chondrogenesis of MSCs (O'Conor et al. 2013; Schatti et al. 2011), while tensile shear forces are important determinants for osteogenic differentiation (Chen and Jacobs 2013; Knothe Tate et al. 2008; Yourek et al. 2010). With this realization that the ECM microenvironment can influence stem cell fate (Hadden and Choi 2016), methods have been developed to engineer the physical environments of MSCs in culture systems to

direct their differentiation in regenerative applications in repair biology (Ahmed and Ffrench-Constant 2016; Brafman 2013; Crowder et al. 2016; Han et al. 2014; Hao et al. 2015) and high throughput screening procedures have been developed to evaluate the stem cell niche and how this is affected by such procedures (Ghaemi et al. 2013).

Like other mammalian cells, MSCs contain a single primary cilium (Satir and Christensen 2007, 2008), however, its function is relatively poorly understood (Nathwani et al. 2014). In other cell types, the primary cilium (Mahjoub 2013) co-ordinates transcription factor responses arising from mechanical stimuli (Liu et al. 2014; Nachury 2014), integrating these into biochemical responses through receptor tyrosine kinase-mediated cell signalling (Liu et al. 2014; Christensen et al. 2013). The primary cilium is a sensory microtubule-encased membranous protrusion from the cell surface, which regulates the cell cycle, cell proliferation and embryonic development (Ke and Yang 2014) and directional migration of fibroblasts (Christensen et al. 2013). In stem cells, the microtubular structure of the primary cilium and its connection with the centriole is consistent with a probable cell division regulatory function (Ke and Yang 2014) and cell signalling responses to mechanical stimuli (Liu et al. 2014).

4.8 CS Bioscaffolds and Their Instructional Properties Over MSC Differentiation

Two studies have recently reviewed the instructive properties of CS bioscaffolds and how they direct the differentiation of chondrogenic, osteogenic and neurogenic progenitor cells and how they have been applied in tissue repair strategies (Andrews et al. 2019; Farrugia et al. 2018). A few representative examples of these CS bioscaffolds are provided in Table 4.3.

The three-dimensional cues provided by integrated biomaterials potentiate differentiation of hMSCs in vitro (Park et al. 2018). A comparative study of CS and HS to promote three-dimensional chondrogenesis of MSCs showed that CS-containing hydrogels at low mechanical stiffness provided a promising scaffold for enhancing MSC-based cartilage tissue regeneration. CS was more potent at inducing chondrogenesis than HS (Wang and Yang 2017). Gelatin micro-ribbon hydrogels accelerate chondrogenesis by MSCs in three dimensions (Conrad et al. 2018). 3D composite bioscaffolds of polyethylene glycol (PEG) and CS have been prepared in an attempt to reproduce an MSC stem cell niche microenvironment to promote chondrogenic differentiation of MSCs and in vitro chondrogenesis (Varghese et al. 2008). 3D bio-printing of biomimetic scaffolds (Costantini et al. 2016) and biomimetic hydrogels (Liu et al. 2010) seeded with bone marrow MSCs have also been evaluated as a means of producing neocartilage in cartilage repair biology. The cellular microenvironment in scaffolds and paracrine signals are important functional determinants that influence chondrogenic differentiation (Grassel and Ahmed 2007). Chondrogenic differentiation of MSCs embedded in a scaffold has been

Table 4.3 Evaluation of the instructive properties of chondroitin sulphate (CS) in bioscaffolds designed to regulate progenitor stem cell differentiation

Scaffold	Scaffold properties	References
Zonally tailored CS scaffolds	Osteochondral differentiation of synovial MSCs	Diaz-Rodriguez et al. (2018)
Nano-microspheres containing chitosan, hyaluronic acid and CS	Gene delivery of GDF-5 plasmid for the treatment of osteoarthritis	Chen et al. (2018)
Dynamically loaded cartilage mimetic CS-hydrogel	Regulation of hypertrophy during MSC chondrogenesis	Aisenbrey and Bryant (2019)
Silk fibroin/chitosan-CS 3D scaffolds	Enhanced chondrogenesis of MSCs under dynamic culture conditions	Agrawal et al. (2018b)
CS and HS 3D chondrogenesis cultures	3D culture of MSCs ± CS or HS	Wang and Yang (2017)
CS-based biomineralizing hydrogels	Bone tissue engineering	Kim et al. (2017)
Microspheres containing encapsulated CS or decellularized cartilage	Poly D,L-lactic-co-glycolic acid encapsulated scaffolds promote chondrogenesis	Gupta et al. (2016)
Self-Assembling CS/Dec bioscaffolds	Cartilage tissue engineering	Recha-Sancho and Semino (2016)
GAG-Collagen I multilayers	Assessment of osteogenic differentiation of adipose MSCs	Zhao et al. (2016)
CS immobilized on a biomimetic scaffold	Modulation of inflammation during promotion of chondrogenesis	Corradetti et al. (2016)
Sericin loaded electrospun nanofibrous composite scaffold (cationic gelatin-HA-CS)	Co-cultivation of keratinocytes and hMSCs to stimulate epithelial cell differentiation by hMSCs	Bhowmick et al. (2016)
Natural-synthetic hybrid hydrogels	Tunable growth factor delivery for promotion of MSC differentiation	Anjum et al. (2016)
Biomimetic microbeads containing a CS-chitosan polyelectrolyte complex	Development of cell-based cartilage therapies	Daley et al. (2015)
Composite hydrogel of chitosan-poly hydroxybutyrate-co-valerate with CS nanoparticles	Tissue engineering for nucleus pulposus tissue	Nair et al. (2015)
Composite methacrylated CS hydrogels	Assessment of decellularized adipose tissue particle size and cell density on adipose stem cell proliferation and differentiation	Brown et al. (2015)
Silk fibroin/gelatin-CS-HA composite bioscaffolds	Enhancement of bone marrow-derived stromal MSC chondrogenesis	Sawatjui et al. (2015)
highly porous CS supplemented alginate-foams	Enhanced chondrogenesis of bone marrow-derived stromal MSCs	Huang et al. (2015)
Elastic HA-CS hydrogel	Tough support matrix for the culture of MSCs	Ni et al. (2015)

(continued)

Table 4.3 (continued)

Scaffold	Scaffold properties	References
Immobilized lentivirus vector on CS-HA-silk fibroin hybrid scaffold	Specific application for the tissue engineering of the ligament–bone junction	Sun et al. (2014)
Novel oxidized CS surface coating BMP delivery system	BMP-2 delivery system for bone regeneration	Anouz et al. (2018)
CS and HA nanofibres	Nanofibre alignment effects on bone marrow MSCs in cartilage repair	Lee et al. (2014)
Bioscaffolds containing oversulphated GAGs including CS	Promotion of the differentiation of osteoblast-precursor cells and pre-mature osteoblasts	Hempel et al. (2014b)
Assembly of star-shaped CS structures of increased bioactivity mimicking proteo-glycan structure	Star-shaped GAGs form fibres with growth factors with improved deliv-ery properties	Novoa-Carballal et al. (2018)
CS and HA doped conductive polypyrrole films	Culture of adipose stem cells	Bjorninen et al. (2014)
CS 3D scaffolds containing multi-walled carbon nanotubes	Nervous tissue repair	Serrano et al. (2014)
Chitosan-CS nanoparticles for controlled delivery of platelet lysates in bone regenerative medicine	Application of controlled delivery of platelet lysates in bone regenerative medicine	Santo et al. (2012)
CS-PEG hydrogels	Assessment of dynamic loading and the effect of CS content of hydrogels on chondrogenesis by MSCs	Steinmetz and Bryant (2012)
Nano- and microscale CS particles for controlled growth factor delivery	Optimization of growth factor delivery in chondrogenesis	Lim et al. (2011)
TGF-β3 PLGA-gelatin-CS-HA hybrid scaffold	Promotion of cartilage regeneration	Fan et al. (2010)
Slow extended release of TGF-β3 complexed with CS in bioscaffolds	Improved MSC chondrogenic differentiation	Park et al. (2010)
Injectable cross-linked CS	Cross-linked CS hydrogels for car-tilage and bone repair	Bai et al. (2017)
Microsphere gradient implants	Osteochondral regeneration	Mohan et al. (2015)
Aligned CS and HA on nanofibres	Stimulation of bone marrow MSCs for cartilage regeneration	Lee et al. (2014)
ECM inspired coatings on 3D scaffolds	Calvarial bone regeneration	Rentsch et al. (2014)

induced by long-term release of TGF-β3 complexed with CS (Park et al. 2010). Sustained release of TGF-β1 by genetically-modified cells induces chondrogenic differentiation of MSCs (Askari et al. 2018). TGFβ1 is involved in the chondrogenic differentiation of MSCs and is dysregulated in OA (Ruiz et al. 2018). CS regulates chondrocyte hypertrophy in a cartilage mimetic hydrogel scaffold under dynamic loading (Aisenbrey and Bryant 2019). Cartilaginous ECM components in HA hydrogels influence chondrogenic differentiation of MSCs and chondrocyte hyper-trophy (Zhu et al. 2017). Hypertrophic differentiation of MSCs is suppressed by

xanthotoxin (Cao et al. 2017). MSC hypertrophy and endochondral ossification are also suppressed in 3D cartilage mimetic nanofibrous PLLA scaffolds containing matrilin-3 (Liu et al. 2018). PTHrP gradients affect the progression of chondrogenesis mediated by MSCs and the development of hypertrophy (Fahy et al. 2018), PTHrP has been used in combination with FGF-2 and TGFβ1/BMP2 to improve chondrogenic differentiation of MSCs and regulate hypertrophy (Nasrabadi et al. 2018).

A number of innovative bioscaffold designs have been employed using CS as a component to modulate the differentiation of stem cells for improved repair or regeneration of cartilaginous and nervous tissues and bone. These include zonally tailored CS scaffolds (Diaz-Rodriguez et al. 2018), composite nano-spheres (Chen et al. 2018), microspheres, microbeads, cartilage mimetic CS hydrogels (Aisenbrey and Bryant 2019), silk-fibroin-chitosan-CS bioscaffolds (Agrawal et al. 2018a, b), CS biomineralizing surface hydrogels (Kim et al. 2017), self-assembling CS scaffolds (Recha-Sancho and Semino 2016), GAG-collagen multilayers (Zhao et al. 2016), electrospun nanofibrous gelatin-HA-CS composites (Pezeshki-Modaress et al. 2016; Brunelle et al. 2018), methacrylated CS hydrogels, highly porous CS supplemented alginate-foams (Huang et al. 2015), elastic HA-CS hydrogels, lentivirus vector immobilized on CS-HA-silk fibroin hybrid scaffolds (Sun et al. 2014), CS and HA nanofibres, CS and HA doped electro-conductive polypyrrole films (Bjorninen et al. 2014), CS 3D bioscaffolds containing multi-walled carbon nanotubes (Serrano et al. 2014), controlled delivery platelet lysate or growth factor delivery nanoparticles (Busilacchi et al. 2013; Santo et al. 2012). Examples of these applications are listed in Table 4.3.

4.9 Expression of the 7-D-4, 3-B-3(−) and 4-C-3 CS Sulphation Motifs Are Upregulated During Osteogenic Differentiation and Stimulated by FGF-18

Micro-mass pellet cultures of bone marrow stromal mesenchymal stem cells stimulated with FGF-18 undergo osteogenic differentiation in vitro. This correlates with the immunolocalization of the 7-D-4, 4-C-3 and 3-B-3(−) CS sulphation motifs at the bone-cartilage interface of growth plates undergoing endochondral ossification and with the prominent immunolocalization of FGF-18 by hypertrophic growth plate chondrocytes (Table 4.4).

4.10 Concluding Remarks

CS in all its isomeric forms is an important repository of molecular recognition and information which is deciphered by progenitor cells and conveys important directive cues over cellular behaviour in proliferation and differentiation and tissue expansion

Table 4.4 CS sulphation motifs identified in specific proteoglycans

Proteoglycan	CS Motifs Identified	References
	3-B-3(+)/2-B-6 (+)	Caterson et al. (1985), Melrose et al. (1998), Shu and Melrose (2018)
Aggrecan	3-B-3(−)	Farrugia et al. (2019)
	4-C-3	Hayes et al. (2011), Shu et al. (2013)
	7-D-4	Hayes et al. (2011), Shu et al. (2013), Melrose et al. (2000, 2001)
	3-B-3(+)/2-B-6 (+)	Caterson et al. (1985)
Perlecan	2-B-6(−)	Farrugia et al. (2016b)
	4-C-3	Hayes et al. (2011), Shu et al. (2013)
	7-D-4	Hayes et al. (2011), Shu et al. (2013), Melrose et al. (2000, 2001)
	3-B-3(+)/2-B-6 (+)	Caterson et al. (1985), Melrose et al. (1998), Shu and Melrose (2018)
Decorin	4-C-3	Shu et al. (2016)
	7-D-4	Shu et al. (2016), Melrose et al. (2000, 2001)
	3-B-3(+)/2-B-6 (+)	Caterson et al. (1985)
Biglycan	4-C-3	Shu et al. (2016)
	7-D-4	Shu et al. (2016), Melrose et al. (2000, 2001)
	2-B-6(−)	Farrugia et al. (2016b)
Serglycin	3-B-3(−)	Farrugia et al. (2019)
	3-B-3(+)/2-B-6 (+)	Caterson et al. (1985)

during tissue development. The modulation of GAG substructure during tissue remodelling and skeletal development is an important example of the dynamic cell regulatory properties which proteoglycans convey to regulate tissue form and function. A greater understanding of how progenitor cells interpret this cell directive glyco-code would be extremely useful in tissue repair strategies aimed at recovering functional properties in recalcitrant tissues such as degenerate articular cartilage or ulcerated diabetic skin wounds, both of which have extremely limited self-repair capability.

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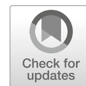
Chapter 5
Regulatory Functions of Heparan Sulfate in Stem Cell Self-Renewal and Differentiation



Sumit Rai and Lianchun Wang

Abstract Heparan sulfate (HS) is an extraordinarily diverse type of glycosaminoglycan (GAG) that is ubiquitously expressed on the cell surface and in the extracellular matrix where it interacts with a multitude of growth factors and morphogens. The association of HS chains with the various protein partners, though seemingly promiscuous, is surprisingly unique, and is largely dictated by the spatiotemporal expression of various modifying enzymes. Playing a central role in a myriad of cellular and physiological processes, its involvement in regulating developmental decisions is under intense investigation. Stem cells are distinguished by their characteristics of self-renewal and pluripotency. Self-renewal allows stem cells to proliferate indefinitely in their undifferentiated/early differentiated state, whereas pluripotency implies their capacity to differentiate into different cell lineages. Recent studies have provided important information regarding the role of HS in regulating the self-renewal and differentiation capacity of both embryonic and adult stem cells. Here we review the current advances that have been made in understanding the function and related structural changes of HS during stem cell differentiation and in deciphering the underlying molecular mechanisms with a focus on embryonic stem cells (ESCs), neuronal progenitor cells (NPC), and prostate stem cells (PrSCs).

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5.1 Introduction

Mammalian embryogenesis is a highly orchestrated developmental process that begins with the formation of the zygote, resulting from subsequent sperm–egg fusion. The zygote gives rise to the embryo and all the extraembryonic cell lineages required to sustain proper development, a property termed as totipotency (Condic 2014). The zygote retains this totipotent state until the blastocyst stage is reached after which the cells gradually start to undergo differentiation losing their developmental potency. The blastocyst consists of an outer layer of trophectoderm (TE) and an inner cluster of cells known as the inner cell mass (ICM). As development proceeds, the homogenous cell population of the ICM is segregated into the primitive endoderm (PE) and the epiblast (EPI) by the end of implantation (Fig. 5.1a). It was shown that ICM exhibits a random "salt and pepper" pattern of expression of the Nanog and Gata family of transcriptional factors in a nonoverlapping fashion. The Nanog$^+$ subpopulation of the ICM gives rise to the epiblast while Gata6$^+$ cells differentiate to PE (Chazaud et al. 2006). PE predominantly gives rise to the cells of extraembryonic lineage while ICM develops into the fetus and forms some of the extraembryonic tissues (Gardner 1982, 1984; Gardner and Rossant 1979; Plusa et al. 2008). Embryonic stem cells (ESCs) are derived from the ICM of the preimplantation blastocyst and are pluripotent in nature, i.e., contribute to the three germ layers and the germline of the embryo (Evans and Kaufman 1981; Martin 1981). Following the establishment of the mouse ESC (mESC) line, human ESCs (hESC) were derived from the human blastocyst (Martin 1981; Thomson et al. 1998). These ESCs are easily expanded indefinitely in vitro to give rise to a homogenous population of undifferentiated cells (Smith and Hooper 1987). In recent years, tissue/organ-specific stem cells (progenitor cells) have been also identified. In contrast to primary cells which undergo senescence after a finite number of cell divisions, stem cells appear to be immortal. Stem cells retain their pluripotent potential through a process of self-renewal which makes them a tractable model to study early embryonic/tissue development and aid in investigating cell fate decisions with a promise to develop effective treatments for cell and tissue therapy in regenerative medicine.

Even though ESCs have the ability to give rise to various cell types or tissue-specific stem cells to differentiate into mature cells, our ability to direct stem cells to differentiate into specific developmental pathways and promote maturation of individual differentiated phenotypes, though under immense investigation, is not yet well established. A large body of research has determined a comprehensive list of factors that are intimately associated with regulating a tight balance between stem cell self-renewal and lineage specification. These include a variety of growth factors, cytokines, chromatin modifiers as well as transcriptional and posttranscriptional mechanisms. The extent of differentiation as well as the proportion of the differentiated product is greatly influenced by the relative strength of extrinsic cues, which these stem cells are subjected to, and the interconnected circuitry of the signaling pathways that works to aid the self-renewal process. ESCs cultured in serum-containing medium as an adherent monolayer, in the absence of antidifferentiation

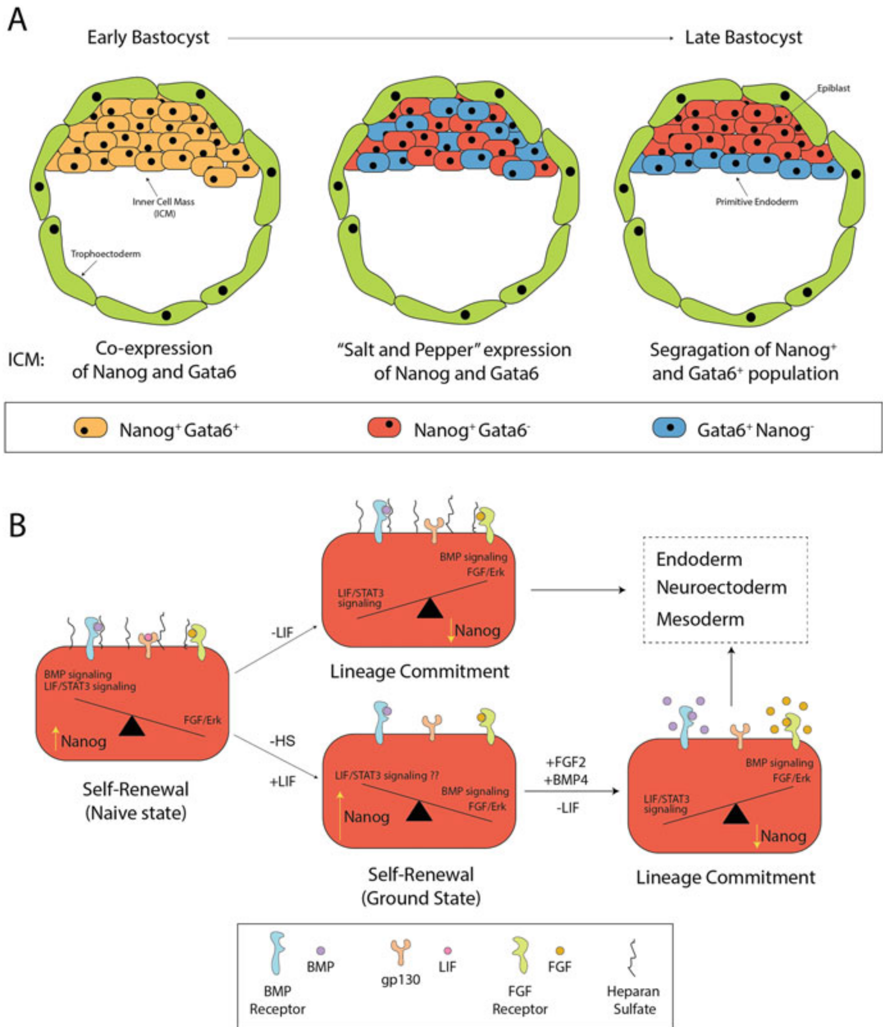

Fig. 5.1 ES cell fate commitment. (**a**) Cells of the inner cell mass (ICM) display an overlapping expression of lineage-specific transcription factors (Gata6 and Nanog). The ICM further exhibits a "salt-and-pepper" pattern of epiblast cells (Nanog$^+$) and primitive endoderm (Gata6$^+$) which eventually are spatially segregated at the late blastocyst stage. (**b**) HS modulates FGF and BMP4 signaling to dictate ESC self-renewal and cell lineage commitment. Upon LIF withdrawal, HS facilitates FGF signaling which decreases Nanog expression to allow for multilineage differentiation. HS deficient mESCs are arrested in the "ground state" of pluripotency in the absence of differentiating FGF/Erk signals exhibiting a differentiation block. After exiting from self-renewal, HS deficient mESCs do not undergo mesodermal differentiation, a defect that can be restored by exogenous supplementation of high levels of FGF2 and BMP4

factors, undergo spontaneous differentiation generating a milieu of different cell types. When a similar differentiation protocol is applied to ESCs cultured in suspension, they spontaneously differentiate to form three-dimensional multicellular aggregates called Embryoid Bodies (EBs). EBs recapitulate many molecular and cellular events associated with early lineage establishment and have served as an effective tool in providing a plethora of information regarding the highly intricate processes that govern the seemingly simple embryonic development. Investigations into the mechanisms of ESC differentiation have provided insights into devising direct differentiation protocols that allow differentiating ESCs to a particular lineage or cell type (Keller 2005; Wobus and Boheler 2005). Despite recent advancements, a major challenge in the field remains: to achieve stable and homogenous differentiation of stem cells into cell types that may eventually be used for curative purposes. New insights into the complexities of cell signaling and the molecular mechanisms that control stem cell fate are necessary to improve the efficacy of directed stem cell differentiation.

Heparan sulfate (HS) is a highly sulfated unbranched complex polysaccharide that is found on the cell surface and in the extracellular matrix where it allows for the interactions between cells and their surrounding environment (Bernfield et al. 1999; Bishop et al. 2007). The HS chains are present in all of the mammalian tissues and are intimately associated with a multitude of cellular and physiological processes by virtue of their ability to interact with a plethora of proteins. Their promiscuity with interacting partners enables HS chains to serve various functions, including acting as co-receptors in various signaling reactions or affecting the distribution of chemokine and morphogens in the vicinity (Balasubramanian and Zhang 2016; Bishop et al. 2007; Jakobsson et al. 2006; Rapraeger 1995). Since HS is at the heart of various developmental processes, its regulatory importance in affecting stem cell functions is paramount. Functional studies have shown that HS is required not only for the initial specification of the germ layers but also for the formation of progenitors or terminally differentiated cells like hemangioblasts, endothelial cells, adipocytes, and neurons (Forsberg et al. 2012; Holley et al. 2011; Jakobsson et al. 2006; Johnson et al. 2007; Kraushaar et al. 2013; Lanner et al. 2010). In this review, we focus to collate recent studies which have dissected the molecular mechanisms of HS in regulating ESC, NPC, and PrSC functions and related signaling.

5.2 Heparan Sulfate

HS belongs to the family of glycosaminoglycans (GAG). It is composed of glucuronic acid (GlcA)/iduronic acid (IdoA) residues and *N*-acetyl glucosamines (GlcNAc) with various sulfation modifications and is typically 50–200 disaccharides in length. The biosynthesis of HS is a complex process involving at least 20 HS-specific biosynthetic enzymes (Fig. 5.2). The first step of HS biosynthesis involves the formation of a tetrasaccharide linker composed of GlcAβ(1→3)Galβ(1→3)Galβ(1→4)Xylβ1 at specific serine residue/s of a core protein (Lindahl et al.

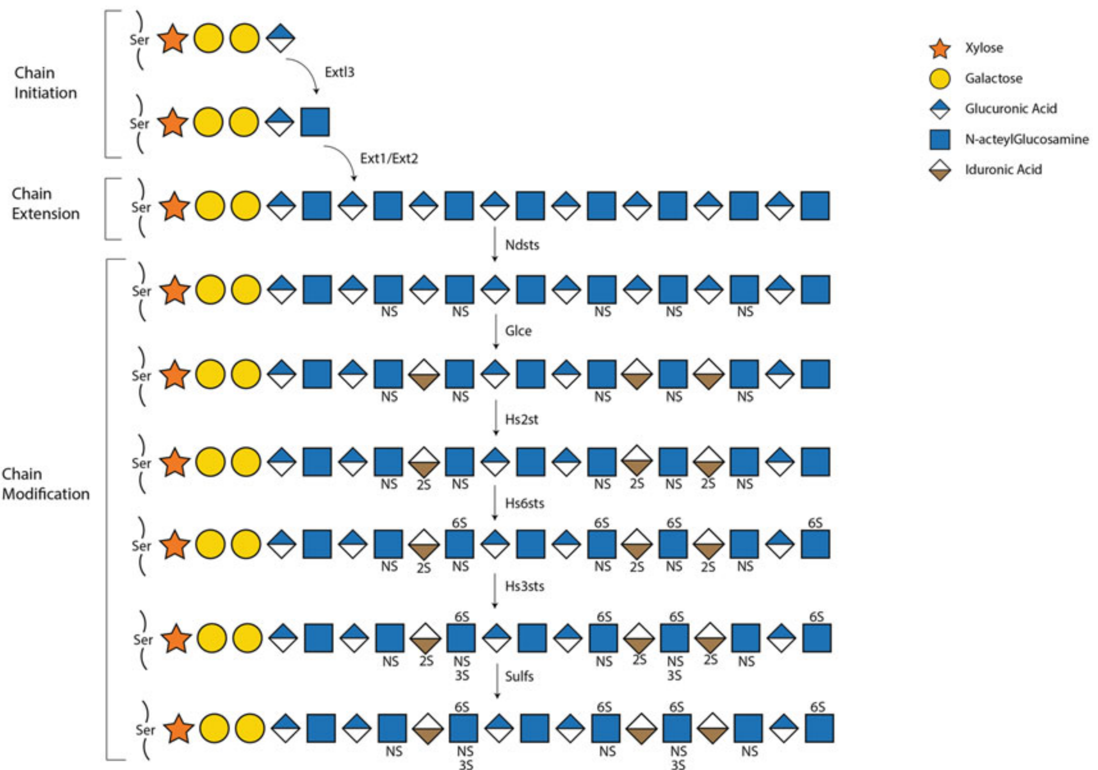

Fig. 5.2 Biosynthesis of heparan sulfate chain. Heparan sulfate (HS) chains are covalently linked to a specific serine residue of a core protein from which a tetrasaccharide linker extends. The addition of the first *N*-acetylglucosamine marks the chain initiation step which is catalyzed by the EXTL3 enzyme. A heterodimer of EXT1 and EXT2 elongates the chain by alternate addition of glucuronic acid and *N*-acetylglucosamine. As the chain is being polymerized, a number of enzymes modify the HS chains through epimerization and sulfation reactions at the various positions. Ser, serine; NS, *N*-sulfate group on glucosamine residues; 2S, 2-*O*-sulfate group on glucuronic or iduronic acid residues; 3S, 3-*O*-sulfate group on glucosamine residues; 6S, 6-*O*-sulfate group

1965; Zhang et al. 1995; Zhang and Esko 1994). Following, Exostosin-like 3 (Extl3) encodes the α1,4-*N*-acetylhexosaminyltransferase that transfers GlcNAc to the tetrasaccharide linker initiating HS polymerization, differentiating it from the chondroitin sulfate (CS)/dermatan sulfate synthesis (Kitagawa et al. 1999; Wuyts et al. 1997). After the addition of the GlcNAc residue, polymerization of the HS backbone is initiated by the alternate addition of β1,4-linked GlcA and α1,4-linked GlcNAc units to the nonreducing end of the growing polymer by co-polymerases Exostosin 1 (Ext1) and Exostosin 2 (Ext2), which are bifunctional glycosyltransferases harboring both GlcA- and GlcNAc-transferase activity (Lind et al. 1998; McCormick et al. 2000; McCormick et al. 1998; Senay et al. 2000). As the HS chain is being extended, the *N*-deacetylase *N*-sulfotransferases (Ndst) substitute the *N*-acetyl groups with *N*-sulfate groups of some GlcNAc residues. The four isoforms of Ndst (Ndst1-4) exhibit striking differences in the proportion of N-deacetylation and N-sulfation activities (Aikawa et al. 2001). These N-sulfated regions undergo further modifications by a number of enzymes including epimerization of GlcA to IdoA by C5-epimerase (Glce), advancing toward O-sulfation at various positions by iduronosyl 2-*O*-sulfotransferase (HS2st), glucosaminyl 6-*O*-sulfotransferases (HS6sts), and 3-*O*-sulfotransferases (HS3sts). Three isoforms of HS6st and seven isoforms of HS3st have been described, whereas only one isoform of Glce and HS2st exists. These modification steps are highly regulated with enzymes using products of preceding enzymatic reaction as substrates. However, certain in vitro and cell-based studies have revealed otherwise. Modifications of the HS structure, though mostly occurring in the Golgi, are not restricted within the cell. The HS chains are post-synthetically remodeled at the cell surface by extracellular endosulfatases (Sulf), which can remove 6-*O* sulfates from GlcNAc residues (Uchimura et al. 2006). These HS chains can also be degraded by an endoglycosidase, heparanase (Sanderson et al. 2017). There are two isoforms of sulfatase and heparanase. The sulfation and epimerization modifications, both intra- and extracellularly, are not evenly spread across the HS chains giving rise to variable HS sequences. For example, Ndsts give rise to segments with unmodified *N*-acetylated disaccharide units (NA domains), sequences of *N*-sulfated disaccharide units (NS domains), and regions composed of alternating *N*-sulfated and *N*-acetylated disaccharides (NA/NS domains) (Murphy et al. 2004). Thereby, a vast number of highly variable HS sequences are generated. Interestingly, these modifications are not random and have been shown to be under tight regulation in a context-specific manner. Since the sequence and sulfation pattern of HS chains dictates interaction with a plethora of binding partners, these modifications are believed to fine-tune the spatiotemporal regulatory functions of HS during development and in disease progression (Bishop et al. 2007; Esko and Selleck 2002; Rapraeger 2002).

Fig. 5.2 (continued) on glucosamine residues; Extl3, exoston-like 3; Ext1, exostosin 1; Ext2, exostosm 2; Ndst, *N*-deacelylase/*N*-sulfotransferase; Glce, glucuronyl C5-epimerase; Hs2st, HS 2-*O*-sulfotransferase; HS6st, HS 6-*O*-sulfotransferase; HS3st, 3-*O*-sulfotransferase; Sulf, sulfatases

5.3 Heparan Sulfate Expression During Embryonic Stem Cell Self-Renewal and Differentiation

The HS chain structures on stem and progenitor cells appear extremely dynamic in nature. Spatiotemporal regulation of the various enzymes orchestrating HS biosynthesis remodels the HS chain structure as cells advance through the various developmental stages. Determination of HS structure and content along with the landscape of HS biosynthetic enzyme gene expression in both undifferentiated and differentiated ESCs has led to a better understanding of the developmental remodeling process. HS chains have been found to be in high abundance on mESC surface, whereas CS chains were found to be highly reduced (Lin et al. 2000; Nairn et al. 2007; Sasaki et al. 2008). Differentiating mESCs exhibit increased HS content as well as sulfation, regardless of the lineage fate. This, however, is not mirrored by transcript levels of the *Ext* family, which point toward the involvement of other enzyme regulation mechanisms. Similarly, in hESCs, HS chain initiation and elongation enzymes did not show any considerable change at the transcript level following differentiation but gained significantly increased sulfation modifications (Gasimli et al. 2014). mESCs and hESCs both showed an increase in the majority of HS as well as CS/DS core proteins on differentiation (Gasimli et al. 2014; Nairn et al. 2007).

HS chains from undifferentiated ESCs are largely undersulfated; HS chains from mESCs are around 30% N-sulfated while hESC HS chains are about 15% N-sulfated (Gasimli et al. 2014; Nairn et al. 2007). Interestingly, gene expression of some of the HS chain modifying enzymes is substantially increased in a lineage-specific manner. These modifications probably tailor the HS chain structure in order to interact selectively with the various morphogens and/or the surrounding ECM critical for accomplishing proper differentiation endpoints. For example, during early embryonic development, the Brachyury gene is expressed throughout the primary mesoderm. Exposing EBs to VEGF results in the formation of endothelial progenitors from Brachyury expressing cells (Baldwin et al. 2008). HS4C3 antibody, which selectively detects HS structures that contain both *N*- and 6-*O*-sulfate groups and a critical 3-*O*-sulfate moiety, exhibited a high binding affinity toward a subpopulation of mesodermal cells. This $HS4C3^{higher}$ fraction was found to bear increased hematopoietic differentiation potential. Additionally, E7.5 mouse embryo staining showed that the HS epitope recognized by HS4C3 is synthesized exclusively by the embryonic mesoderm during gastrulation (Baldwin et al. 2008). This suggests that the HS structures are continuously being remodeled and are under tremendous regulatory control in a lineage-specific manner. Similarly, as ESCs commit to differentiation, there is an increase in N-, 3-O-, and 6-O-sulfation due to lineage-specific increases in various HS sulfotransferases. For instance, *HS3st1* expression is reported in the heart, brain, and kidney, whereas *HS3st2* is expressed only in the brain (Shworak et al. 1999). In agreement with this finding, the transcript level of *HS3st1* is increased, whereas the level of *HS3st2* is decreased in differentiating hESCs to splanchnic mesoderm (Gasimli et al. 2014). In another example, $Sox1^+$

neural cells derived from mESCs demonstrate significant increases in *Ndst4*, *HS3st3a*, and *HS3st5* transcript levels, which are typically expressed in the fetal brain (Johnson et al. 2007; Mochizuki et al. 2003; Yabe et al. 2005). In contrast, differentiation to extraembryonic cell types is accompanied by increased *Ndst1*, *Ndst2*, *HS6st2*, and *Hs3sta1* transcript levels (Nairn et al. 2007). In summary, current data support a concept that HS expression appears to be upregulated during differentiation and organ/cell-type/development stage-specific at both HS gene expression and HS structural levels.

5.4 Heparan Sulfate Regulates ESC Self-Renewal and Related Signaling

The role of HS in regulating cell fate decisions of ESCs has been investigated by generating mESC lines that harbor mutations/suppressions in HS enzymes, including the generation of *Ext1* null ($Ext1^{-/-}$), $Ext1^{cn/cn}$ (an mESC line generated through conditional ablation of *Ext1* allele in vitro), *Ext1*-KD generated by transfecting ESCs with siRNAs against *Ext1,* and *Ndst1/2* null ($Ndst1/2^{-/-}$) mESCs (Forsberg et al. 2012; Kraushaar et al. 2010; Sasaki et al. 2008). Surprisingly, the $EXT1^{-/-}$*,* $Ext1^{cn/cn}$, and $Ndst1/2^{-/-}$ mESCs exhibit refractoriness to exit self-renewal. These mutant cells display continued high levels of pluripotent gene expression even in the absence of self-renewing factor LIF, showing that HS is required for mESCs to exit self-renewal (Fig. 5.1b). However, *Ext1*-KD mESCs exhibited spontaneous differentiation and slow proliferation (Sasaki et al. 2008). This could be attributed to the continued expression of HS chains in the *Ext1-KD* mESCs, albeit of reduced length and altered sulfation pattern, due to incomplete depletion of the *Ext1* mRNA. This observation suggests that residual HS of the *Ext1*-KD mESCs express unique HS structures that interact more efficiently with pro-differentiation factors tilting the balance toward differentiation, as opposed to self-renewal. Based on this presumption and the abovementioned HS mutant ESC studies, it is generally believed that HS is dispensable for the maintenance of stem cell self-renewal state, but is required for cells exit from a self-renewal state (Forsberg et al. 2012; Holley et al. 2011; Johnson et al. 2007; Kraushaar et al. 2010; Lanner et al. 2010).

An interconnected circuitry of several different signaling pathways regulate the self-renewal of ESCs. FGF signaling plays an essential role throughout early mouse embryonic development (Fig. 5.1b) (Rappolee et al. 1994). Mouse ESCs express FGF, which activates the FGF/Erk signaling in an autocrine manner. FGF/Erk signaling is required for early differentiation in the mouse blastocyst. Interestingly, this autocrine FGF/Erk renders ES cells responsive to further inductive signals by exiting the self-renewal state (Kunath et al. 2007). It has been reported that LIF supports self-renewal by blocking FGF/Erk signaling (Silva and Smith 2008). Studies have demonstrated that FGF signaling works upstream of the MAP kinase that is necessary for the downregulation of pluripotency genes, including Nanog, and

further lineage commitment (Hamazaki et al. 2006; Kunath et al. 2007; Ying et al. 2008). Given the essential requirement of HS in enhancing FGF signaling, it is not surprising for *EXT1*$^{cn/cn}$, *Ext1*$^{-/-}$, and *Ndst1/2*$^{-/-}$ mESCs to retain their stem cell identity by attenuating FGF/Erk signaling leading to the high expression of Nanog and blockage of exit of self-renewal (Kraushaar et al. 2010; Lanner et al. 2010). In addition to their failure to downregulate Nanog, even in the absence of LIF, the level of Nanog expression in the *EXT1*$^{cn/cn}$ mESCs is higher than its expression in wildtype control mESCs with LIF (Kraushaar et al. 2010), indicating that the loss of HS results in a more naïve ground state than the undifferentiated wildtype mESCs cultured with LIF. Together, these observations demonstrate that HS facilitates FGF signaling to downregulate Nanog expression, thereby priming ESCs to exit from self-renewal and to initiate differentiation (Fig. 5.1b).

BMP signaling, via the Smad effector proteins, cooperates with LIF/STAT3 signaling to maintain mESC self-renewal (Fig. 5.1b). BMP/Smad signaling inhibits differentiation by inducing expression of the Id1 transcription factor, which is required for the maintenance of high Nanog levels during self-renewal (Ying et al. 2003). Interestingly, though *EXT1*$^{-/-}$ mESCs were found to be defective in BMP4 signaling, they demonstrated enhanced self-renewal despite reduced phospho-SMAD and ID1 levels (Holley et al. 2011). The observed phenotype may be explained by the relative contributions of BMP4 and FGF signaling for the maintenance of self-renewal. Recent reports have demonstrated that mESCs can be pushed toward “ground state” when cultured with chemical inhibitors of FGF/Erk signaling, in combination with LIF. These inhibitors replace the requirement of BMP and support long-term in vitro culture (Ying et al. 2008). Hence, a low level of Erk activity is a predominant requirement for self-renewal without requiring BMP4 activity. In fact, the principal role of BMP4 signaling in promoting self-renewal is by the inhibition of Erk signaling (Qi et al. 2004). These observations suggest a path that HS loss could lead mESCs to defective BMP signaling, consequently increased Erk activation, and loss of self-renewal. However, *Ext1*$^{-/-}$ and *Ext1*$^{cn/cn}$ mESCs, even in the absence of LIF, exhibit reduced Erk activation and retain self-renewal capability, and rescue of FGF signaling normalizes the self-renewal exit defect of the *Ext1*$^{cn/cn}$ mESCs. These observations suggested that HS may function as a double-edged sword to regulate Erk signaling in ESCs by modulating BMP and FGF signaling to control the self-renewal of the cells, but the HS-FGF2-Erk signaling axis plays a dominant role.

The Wnt signaling pathway also plays an important role in stimulating self-renewal and regulating the differentiation of ESCs. Wnt antagonists suppressed mESC self-renewal; further, this phenotype was rescued by the exogenous supplementation of Wnt ligand (ten Berge et al. 2011). The *Ext1*$^{cn/cn}$ mESCs display increased Wnt activity, indicating that HS inhibits Wnt signaling (Kraushaar et al. 2012). This is in contrast with *Ext1*-KD ESCs, in which the Wnt signal was reduced (Sasaki et al. 2008). It is believed that the short HS chains on the cell surface might enhance the “catch” function mode to lead to the downregulation of Wnt signaling in *Ext1-KD*. Glypican 4, an abundant glypican expressed by mESCs, functions as a positive regulator of self-renewal, although it is not yet well-established whether the

HS chains or core protein is the actual mediator of Wnt signaling (Fico et al. 2012). Theoretically, HS deficiency upregulates Wnt signaling, promoting ESCs to exit from self-renewal; however, the *Ext1*$^{cn/cn}$ mESCs display the opposite phenotype. These observations indicate that, similar to HS-dependent BMP signaling, the HS inhibition of Wnt signaling may participate in modulating the self-renewal capacity of ESCs, but the HS-FGF2-Erk signaling axis plays a dominant role.

Other pathways, including Fas signaling, have also been implicated in promoting ESC's self-renewal capacity, which requires the strict presence of 3-O-sulfated HS structures for the activation of this pathway (Hirano et al. 2012). However, how exactly HS regulates Fas signaling requires further elucidation.

5.5 Heparan Sulfate Regulates Embryonic Stem Cell Differentiation and Related Signaling

Studies have also determined the roles of HS in stem cell differentiation and delineated key underlying cellular signaling. *Ext1*$^{cn/cn}$ ESCs were shown to overcome the differentiation block by exposing monolayer cultures to high doses of FGF-2, in the absence of LIF, to circumvent the requirement of HS in promoting FGF signaling (Kraushaar et al. 2013). This treatment restores Erk signaling and promotes differentiation by downregulating Nanog expression, thus allowing for cells to exit their self-renewing state. Under these conditions of forced differentiation, *EXT1*$^{cn/cn}$ ESCs were able to differentiate into ectodermal and endodermal cell types but failed to form the mesoderm (Kraushaar et al. 2012). This was illustrated by the inability of these differentiating cells to express the pan-mesoderm marker, Brachyury, as well as the abnormal expression of genes associated with dorsoventral patterning, which implies the requirement of HS in mESC differentiation into mesoderm (Kraushaar et al. 2012). Additionally, HS depletion, either by knockdown of PAPST or by heparinase treatment, attenuated BMP signaling in mESCs. This finding suggests a possible role of HS in facilitating BMP signaling, which has a well-defined role in dorsoventral patterning of mesoderm during gastrulation (Sasaki et al. 2009). BMP4 signaling induces ventral-posterior mesoderm and inhibits anterior mesoderm, the latter of which gives rise to definitive endoderm (Hemmati-Brivanlou and Thomsen 1995). *Ext1*$^{cn/cn}$ ESCs display reduced expression of posterior mesoderm genes such as *Evx1* and *Mesp1* and overexpress endodermal genes such as *Sox17* and *Foxa2* (Kraushaar et al. 2012). In addition, neuroectodermal differentiation is inhibited by BMP4 (Finley et al. 1999; Kawasaki et al. 2000; Wilson and Hemmati-Brivanlou 1995). Restoration of BMP signaling rescues aberrant mesoderm differentiation and demonstrates that HS facilitates BMP signaling to facilitate mesoderm differentiation (Kraushaar et al. 2012). Similarly, a decrease in mesodermal differentiation with a concomitant increase in neural differentiation was observed when mESCs were exposed to chlorate, which is a potent inhibitor of sulfation on HS chains (Sasaki et al. 2010). Furthermore, *PAPST1* and

PAPST2 knockdown, which lead to decreased HS and CS sulfation, have been reported to accelerate neural differentiation (Sasaki et al. 2009). These observations strongly suggest that a balance between mesodermal and neuroectodermal fate choice is dictated by BMP signaling, which in turn is facilitated by HS. Altogether, HS is a critical component in mediating ESC differentiation into germ-layer type cells via simultaneous potentiation of both FGF and BMP signaling to promote mesoderm formation (Fig. 5.1b).

5.6 Heparan Sulfate Regulates Neural Progenitor Stem Cell Differentiation and Related Signaling

Studies have also demonstrated that HS plays a pivotal role in governing NPC differentiation. Dynamic interplay between the HS biosynthetic machinery and post-synthetic modifications defines the content and structure of HS chains in NPCs and dynamic alterations during differentiation. These alterations change HS's affinity for morphogens and growth factors, thus guiding the cells toward their final developmental stage. HS on the NPC surface exhibit decreased FGF-2 binding in contrast to HS from ESCs, although NPCs show increased HS modification genes' expressions including *Ndst1-4*, *Hs6st2-3, Hs3st3a*, and *Hs3st5* (Johnson et al. 2007). This suggests that the differentiation of ESCs to NPCs might be partly attributed to increasing sulfation modifications. Interestingly, the further differentiation of NPCs into neuronal phenotype is accompanied by an enhancement of 6-O-sulfation and in HS chain length. These alterations in HS structure lead to preferential binding of FGF-1 at later neuronal differentiation (Brickman et al. 1998a, b; Nurcombe et al. 1993). This suggests that changes in HS structure initially commit the NPCs to differentiate by gradual decrease in FGF-2 affinity, leading to an eventual switch in the chain structure which selectively binds and potentiates FGF-1 signaling, thereby permitting terminal differentiation. These observations revealed that the regulation of key differentiation signals through the expression of unique HS structures adds another layer of regulatory control in defining the ultimate outcome of NPC differentiation (Allen and Rapraeger 2003).

5.7 Heparan Sulfate Regulates Prostate Development and Prostate Stem Cell Activity

The prostate, found only in mammals, is a male accessory sex gland producing the majority of seminal fluid proteins. Like every other organ, the prostate gland harbors resident PrSCs that divide and give rise to cells in order to repair and replace the damaged or old tissues. The growth and development of the prostate gland, which begins in the fetus, is only accomplished after attaining sexual maturity (Hynes and

Fraher 2004; Prins and Putz 2008; Seifert et al. 2008). Prostate morphogenesis is initiated by the epithelial budding of the urogenital sinus (UGS), wherein urogenital sinus epithelium (UGE) cells proliferate, grow, and invade the surrounding urogenital sinus mesenchyme (UGSM). FGF signaling plays an important role in prostate organogenesis. FGF10 is a critical paracrine factor that is intimately involved in the process (Donjacour et al. 2003; Thomson and Cunha 1999). FGF10 is secreted by the UGSM which binds to FGFR2 (isoform IIIb) expressed at the cell surface of UGE. This interaction on the surface of UGE cells requires the strict presence of 6-O sulfated HS chains (Ashikari-Hada et al. 2004; Mohammadi et al. 2005). The prostatic buds, known to be enriched with PrSCs, were found to express highly tri-sulfated HS (containing 2-O, 6-O, and N-sulfation). These highly sulfated HS chains resulted from reduced expression of *Sulf-1*, an enzyme that removes 6-O sulfation, and high expression of *Hs6st1* from the prostatic buds, both of which contribute to higher 6-O sulfation (Buresh-Stiemke et al. 2012). At the ambisexual UGS phase before gender commitment, *Sulf-1* was found to be highly expressed by the UGSM cells, which gradually taper off as UGS undergoes male-specific sexual commitment. Removal of HS O-sulfation, either by treatment of UGS with sodium chlorate or 6-O-sulfation by over-expressing *Sulf-1,* prevented prostate bud formation, which could be rescued by an exogenous supplementation of heparin, which is a highly sulfated form of HS (Buresh et al. 2010). Thus, efficient remodeling of HS chains on the UGE is required for making UGE receptive to FGF signals that emanate from the UGSM, initiating the sex-specific developmental program. These findings are paralleled by a recent study that demonstrated the role of FGF in supporting PrSC self-renewal utilizing the prostate sphere assay. Inhibiting FGF signals in this assay results in the loss of the PrSCs by increasing differentiation, as well as committing cells to apoptosis (Huang et al. 2015). In our ongoing studies, we carried out PrSC studies and proved genetically that HS is required for the maintenance of PrSC activity and differentiation (unpublished data). Altogether, these studies demonstrate the spatiotemporal remodeling of HS tailoring HS chains initiates specific developmental signaling, such as FGF signaling, in order to dictate maintenance of self-renewal or differentiation of PrSCs.

5.8 Conclusions and Perspectives

Understanding the regulation of stem cells remains one of the research frontiers in the biomedical field. Studies have established the critical regulatory functions of HS in stem cell self-renewal and differentiation and delineated key signaling pathways involved, such as FGF and BMP pathways. Currently, it remains a major challenge to delineate the functionally related fine structures of spatiotemporally expressed HS as well as the unique signaling they regulate. Meanwhile, the regulatory functions of HS in several tissue-specific stem cells, such as NPCs and PrSCs mentioned in this review, have been studied, but a landscape of HS function and related signaling in all major tissue/cell type stem cells remains unclear. Gaining knowledge in future

studies will lead to a better understanding of the precise roles of HS, and its related structure and signaling in stem cell fate decisions, thereby facilitating the establishment of the molecular mechanisms of HS during embryogenesis and development. Comprehension of HS and its signaling mechanisms is highly translational and may contribute to the directed manipulation of stem cell fate toward application for regenerative medicine.

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Chapter 6
Proteoglycans, Neurogenesis and Stem Cell Differentiation

Rachel K. Okolicsanyi, Lotta E. Oikari, Chieh Yu, and Larisa M. Haupt

Abstract Proteoglycans (PGs) are critical components of the cellular microenvironment localised to the cell surface or within the extracellular matrix with a wide variety of functions from early development and throughout life. They are predominantly composed of a core protein to which a number of highly sulfated glycosaminoglycan side chains are attached. PG proteins are highly variable and formed by a complex temporal, posttranslational biosynthesis that confers extensive biological diversity. These glycoproteins are critical contributors and regulators of numerous cellular processes including proliferation, stem cell plasticity and self-renewal, growth factor interaction and cell signalling as well as differentiation and lineage specification. There is great interest in how these multifaceted proteins can be used to understand complex cell and developmental biology as well as potential targets or biomarkers to prevent progression and treat a number of diseases. In combination with stem cell therapies, PGs likely provide exciting targets for a number of therapeutic applications. As these complex proteins have been shown to be involved in a number of critical developmental processes including neurogenesis, this chapter summarises PGs and their influence on neurogenesis and stem cell lineage specification and provides a summary of current models exploring their role in human neurogenesis.

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6.1 Proteoglycans

The local cellular microenvironment, or niche, plays a central role in cell regulation and development. A major component of the niche is the extracellular matrix (ECM), which supplies critical biochemical and physical signals along with structural stability to initiate and sustain cellular functions (Chen 2010; Yin et al. 2018; Yu et al. 2018). The ECM supports a rich signalling environment important for a range of developmental decisions (Rozario and DeSimone 2010; Yin et al. 2018; Yu et al. 2018) including lineage specification, through regulation of growth factor (GF) interactions between the ECM and the cell, subsequently controlling cell replication and differentiation (Chen 2010). In addition, the structure and conformation of the ECM mediates physical linkages with the cytoskeleton and the bidirectional flow of information between the extracellular and intracellular compartments (Rozario and DeSimone 2010; Yin et al. 2018; Yu et al. 2018).

Proteoglycans (PGs) are ubiquitous members of the cellular microenvironment localising within the ECM or found on the cell surface as membrane-bound molecules. PG glycoproteins have a wide variety of functions including structural, enzymatic or cell surface receptor functions contributing to multiple roles in development, cell behaviour and homeostasis through mediating local concentrations of signalling molecules such as cytokines and GFs (Rozario and DeSimone 2010). PGs consist of a core protein to which one or more sulfated unbranched glycosaminoglycan (GAG) side chains are covalently attached at specific sites as they traffic through the Golgi. A coordinated posttranslational biosynthesis results in sulfated GAG chains of variable length and sulfation profile with PGs classified into groups based on the structure of their GAG chains and location. These include the heparan sulfate PGs (HSPGs; membrane-bound glypicans (GPCs; 6 members termed GPC1–6), syndecans (SDCs; 4 members termed SDC1–4); ECM embedded perlecan (PER)) and the ECM-localised chondroitin sulfate (CS) PGs (aggrecan, brevican and neurocan; Guimond et al. 2009; Fig. 6.1). The GAG chain diversity generated through a coordinated biosynthesis of *N*- and *O*-sulfation, chain length and sulfation pattern give rise to the localised specific biological diversity of PGs (Okolicsanyi et al. 2014; Xiong et al. 2014). Indeed, the mediation of many downstream effects such as binding/activation/transport of GFs, cytokines and enzymes are dependent on the type and sulfation pattern of these GAG side chains.

6.2 Neurogenesis

Neurogenesis can occur in the embryo and the adult and can be defined as the process during which the nervous system neural stem cells (NSCs) generate neurons. In the adult, neurogenesis includes the generation of neurons and glial cells. Neurogenesis consists of distinct cellular behaviours, including cell proliferation, followed by cell migration and differentiation (Vinci et al. 2016) with the process of

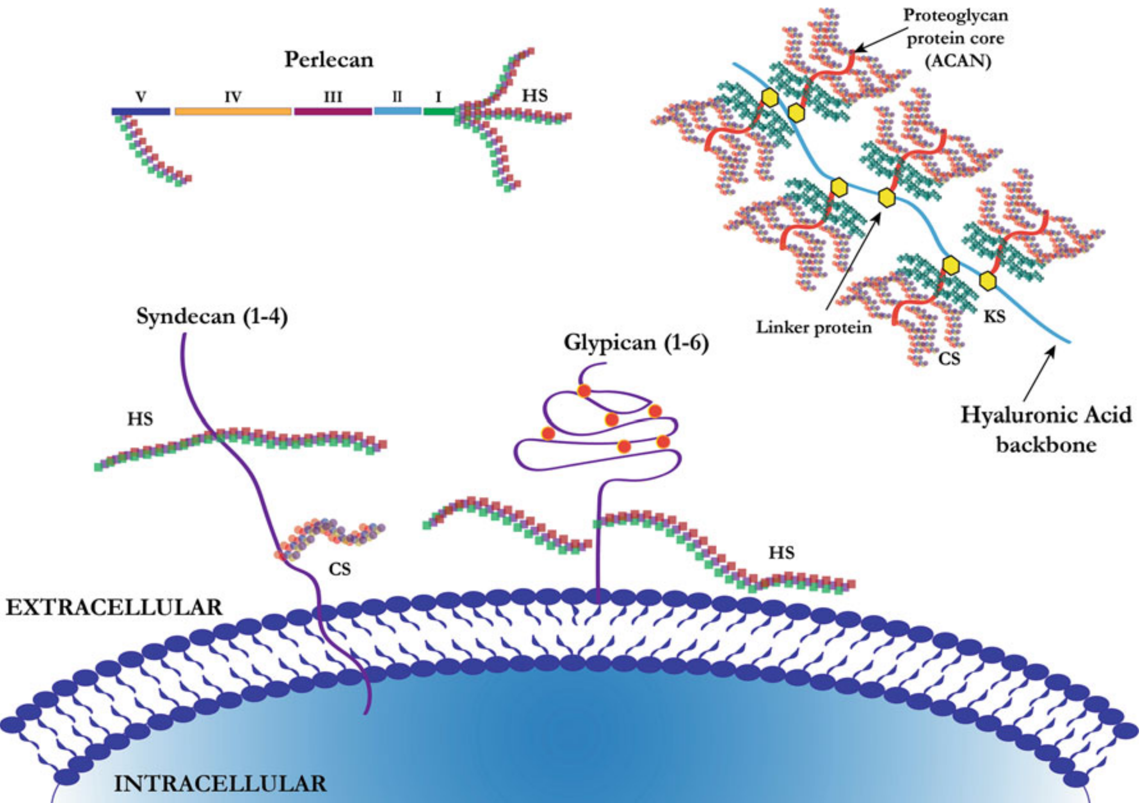

Fig. 6.1 Matrix-bound and extracellular heparan sulfate proteoglycans (HSPGs). Syndecans (SDCs) are a family of four transmembrane proteins that carry predominantly heparan sulfate (HS) chains but can also carry chondroitin sulfate (CS) chains close to the cell surface. Glypicans are a family of six proteins that are attached to the cell membrane through a glycosylphosphatidylinositol (GPI) anchor and carry only HS chains. Hyaluronic acid (HA) is a large protein with bound PGs such as aggrecan (ACAN). Perlecan is an extracellular matrix protein consisting of five domains. HS chains are attached to Domain I and Domain V

neurogenesis differing between the human embryo, the adult human and rodent models.

6.2.1 Human Embryonic Neurogenesis

During embryogenesis, the NSCs—the neuroepithelial (NEP) cells, form the neural tube (Yamashita 2013). All cells of the central nervous system (CNS), including neurons, astrocytes, oligodendrocytes and ependymal cells are produced from these NEP cells (Vinci et al. 2016).

In humans, the production of the neocortex is a temporal and sequential process involving early rapid proliferation followed by a switch from symmetric to asymmetric division of the NEP cells (Budday et al. 2015). Asymmetric division generates one retinal ganglion cell (RGC) with the other daughter cell becoming either a postmitotic neuron or an intermediate progenitor, which also eventually generate pairs of postmitotic neurons (Budday et al. 2015; Silbereis et al. 2016).

The first half of gestation essentially generates large numbers of neural cells, initially mainly neuronal, with the production of glial cells beginning in the second half of gestation (Budday et al. 2015; Silbereis et al. 2016). The predominant activity during the second half of gestation is the development of neural connectivity, which involves axonal extension and formation of connections between neurons (Budday et al. 2015; Silbereis et al. 2016). Synaptogenesis, the formation of synaptic connections, occurs mainly after birth (Budday et al. 2015). While the number of neurons does not increase after birth, axonal myelination and formation of new synaptic connections are characteristics of postnatal brain growth (Budday et al. 2015; Silbereis et al. 2016).

6.2.2 Adult Human Neurogenesis

Identification of the neurogenic areas in the rodent brain (Altman and Das 1965; Kaplan and Hinds 1977; Kaplan and Bell 1984) led to the discovery of neurogenic areas in the adult human CNS. Neurogenesis in the adult human hippocampal dentate gyrus (DG) was demonstrated in the late 1990s through the detection of proliferating cells (Eriksson et al. 1998). Similar expression patterns were observed in the hippocampal regions of both species when neurogenesis-associated markers were examined, including similarities in proliferative markers bromodeoxyuridine/5-bromo-2′-deoxyuridine (BrdU) and neuronal markers (calbindin, NeuN) (Eriksson et al. 1998; Suh et al. 2007). The presence of proliferating cells in the adult human subventricular zone (SVZ) was demonstrated in the early 2000s with these cells, similar to those in the rodent, resembling astrocytes (Sanai et al. 2004).

The difference between species in the structure of the rostral migratory stream (RMS) (Curtis et al. 2007) and differences in the chain-forming ability of neuroblasts

and subsequent chain migration, particularly between foetal and adult human cells (Wang et al. 2011), has lead to the conclusion that while human neurogenesis is known to occur in similar areas to the rodent, it vastly decreases with age and the mechanisms that regulate these processes remain largely unknown (Lim and Alvarez-Buylla 2016).

6.2.3 Understanding Human Neurogenesis: Evidence from Murine and Other Models

Neurogenesis is conserved amongst mammalian species, supported by similar neurogenesis-associated marker expression between rodents and humans. Interestingly, the spatial as well as temporal expression of these markers is also similar between the two models (Knoth et al. 2010). A common characteristic of both rodent and human neurogenesis is its decrease with age; however, the relative decrease is higher in rodents than in humans (Knoth et al. 2010; Spalding et al. 2013). Other reported differences between rodent and human neurogenesis include hippocampal neuron turnover, i.e. the number of new neurons added to the DG to replace missing neurons, which has been reported to be higher in humans than in rodents (Spalding et al. 2013). In contrast, neuron turnover in the human olfactory bulb (OB) is vastly slower than in rodents with reports that in humans less than 1% of OB neurons are exchanged over 100 years while in rodents over 50% of OB neurons are exchanged annually (Bergmann et al. 2012). In addition, a distinct and unique attribute of the human SVZ is a structure termed the astrocyte ribbon, which has not been observed in any other species (Sanai et al. 2004). The human SVZ also contains a unique "gap" region, an area that does not contain cells (Quinones-Hinojosa et al. 2006). Finally, the lack of chain migration in the adult human RMS demonstrates that human neurogenesis contains features that differ from the rodent (Wang et al. 2011). An overview of differences between adult rodent and human neurogenesis is presented in Fig. 6.2. This list is not exhaustive; however, it summarises topics discussed in this chapter.

6.3 Neural Cell Types

Neural cells, including neurons and glial cells, are produced from NSCs during the process of neurogenesis and gliogenesis, respectively. NSCs in the sub-granular zone (SGZ) and SVZ are characterised through the expression of markers including prominin-1 (CD133), nestin (NES), SRY-box transcription factor-1 and -2 (SOX1 and 2), musashi-1 (MSI1), vimentin (VIM) and glial fibrillary acidic protein (GFAP) (Conti et al. 2005; Corti et al. 2007; Sun et al. 2008; Knoth et al. 2010). NSC self-renewal and differentiation (plasticity) is associated with active cell proliferation,

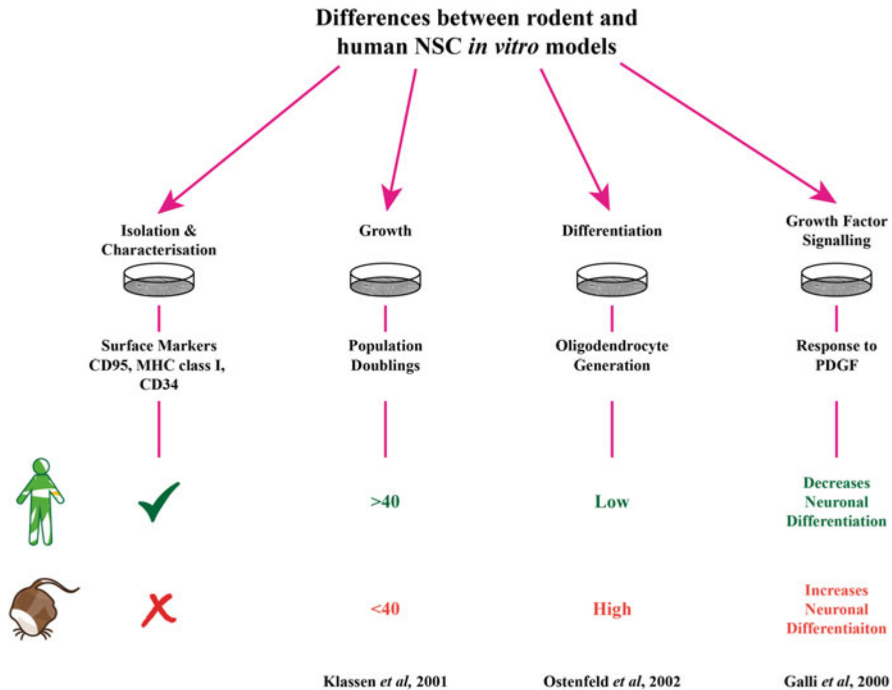

Fig. 6.2 Differences between human and rodent in vitro neural stem cell (NSC) models. Human NSCs display specific characterisation markers to enable isolation and confirmation of cell type. In addition, human cells have a greater in vitro capacity while displaying a decreased capacity for lineage-specific differentiation (adapted from Oikari et al. 2014)

which can be detected through the expression of proliferation markers including BrdU, Ki67, minichromosome maintenance complex component 2 (Mcm2) and proliferating cell nuclear antigen (PCNA) (Eriksson et al. 1998; Curtis et al. 2007; Knoth et al. 2010; Wang et al. 2011). During differentiation, the expression of markers associated with NSC self-renewal switches to the expression of lineage-specific markers, including neuronal, astrocyte and oligodendrocyte markers. Current lineage-specific markers are summarised in Fig. 6.3 and lineage-specific neural cells are discussed in detail below.

6.3.1 Neurons

Neurons are the electrically excitable cells of the CNS that fire action potentials and are responsible for signal transduction in the CNS (Lodish et al. 2000). Neurons maintain a voltage gradient across their membranes via ion pumps with changes in the voltage gradient resulting in the generation of one or many action potentials, which travel along the axon resulting in the activation of synaptic connections

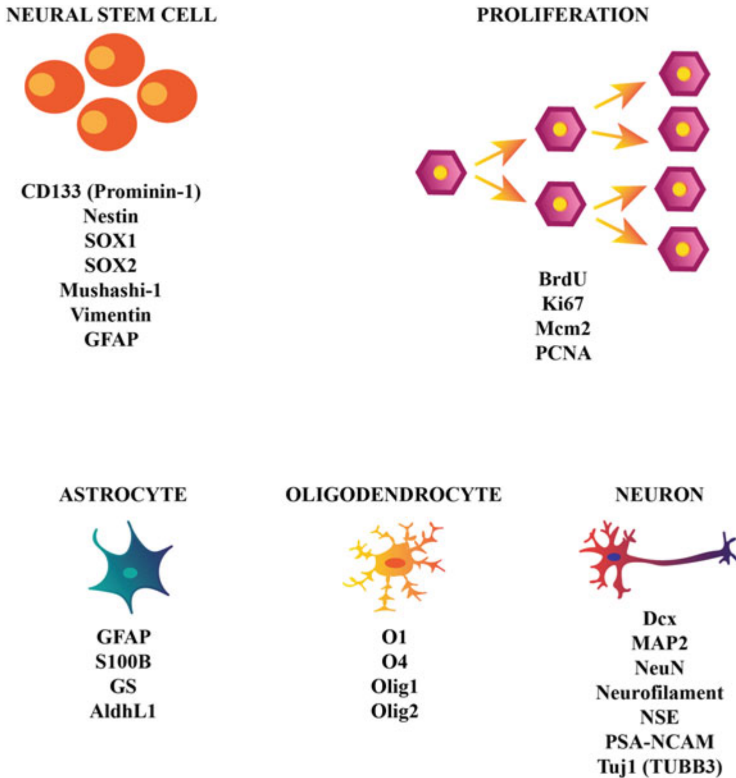

Fig. 6.3 A summary of the markers currently used for the characterisation of rodent and human NSCs and specific neural cell lineages (adapted from Oikari et al. 2014)

(Lodish et al. 2000). In complex organisms, including mammals, neurons are organised in circuits that consist of different neuron types, such as sensory and motor neurons (Lodish et al. 2000). An important characteristic of neurons is membrane trafficking, which plays a key role in providing lipids and membrane proteins for the extending axons and dendrites (Wojnacki and Galli 2016).

Common neuronal cell markers characterised both in the rodent and human include doublecortin (DCX), microtubule-associated protein 2 (MAP2), neuronal nuclei protein (NeuN), neurofilament (NEF), neuron-specific enolase (NSE), polysialylated-neural cell adhesion molecule (PSA-NCAM) and βIII-tubulin (TUBB3) also known as Tuj1 (Eriksson et al. 1998; Doetsch et al. 1999; Sanai et al. 2004; Conti et al. 2005; Sun et al. 2008). Different neuron subtypes also express specific markers, i.e. γ-aminobutyric acid (GABA)ergic, dopaminergic and cholinergic-like motor neurons express GABA, tyrosine hydroxylase (TH) and insulin gene enhancer protein-1/2 (ISL1/2), which are markers of these neurons, respectively (Belinsky et al. 2013; Teratani-Ota et al. 2016).

6.3.2 *Glial Cells: Astrocytes and Oligodendrocytes*

The majority of "electrically silent or non-spiking" brain cells are classified as glial cells, which include at least 50% of all brain cells with the majority of these cells consisting of astrocytes and oligodendrocytes (Rowitch and Kriegstein 2010; Khakh and Sofroniew 2015). Glial cells exist in higher numbers than neurons (approximately 20%) in the brain with 45–75% of these oligodendrocytes and 20–40% astrocytes (Khakh and Sofroniew 2015; Shigetomi et al. 2016).

6.3.2.1 Astrocytes

Mature astrocytes can be divided into two main categories: the "star-shaped" white matter populating fibrous astrocytes; and the highly branched grey matter population of protoplasmic astrocytes (Rowitch and Kriegstein 2010; Sofroniew and Vinters 2010). Common markers expressed by astrocytes include GFAP, calcium-binding protein S100B, glutamine synthetase (GS) and Aldh1L1; however, as astrocytes are a heterogeneous population of cells, some of these markers, including GFAP, are not expressed by all astrocytes (Reeves et al. 1989; Donato 2001; Sofroniew and Vinters 2010; Sosunov et al. 2014). In response to CNS damage and disease astrocytes can undergo functional, morphological and proliferative changes, a process known as reactive astrogliogenesis. This process is responsible for the formation of the glial scar, an area high in ECM proteins that blocks further axonal growth in the area (Khakh and Sofroniew 2015; Fernandez-Castaneda and Gaultier 2016).

6.3.2.2 Oligodendrocytes

Oligodendrocytes are the myelin-producing cells of the CNS and they are generated from highly proliferative oligodendrocyte precursor cells (OPCs), which remain in abundant numbers in the adult CNS and are capable of generating adult oligodendrocytes when needed (Bergles and Richardson 2015). OPCs express amongst others the platelet-derived growth factor receptor-α (PDGFRα), ganglioside A2B5 and proteoglycan NG2 with OPCs also known as NG2 cells (Barateiro and Fernandes 2014). Once OPCs begin to mature towards oligodendrocytes, they start to express lineage-specific markers including galactosylceramidase (GalC), surface markers O1 and O4 and oligodendrocyte transcription factors Olig 1 and Olig 2 (Wegner 2008; Tracy et al. 2011; Barateiro and Fernandes 2014). Structurally oligodendrocytes have multiple extended processes that allow them to form contacts with axons and produce a myelin sheath along the axon (Barateiro and Fernandes 2014). Following CNS injury, OPCs along with reactive astrocytes contribute to the formation of the glial scar (Fernandez-Castaneda and Gaultier 2016).

6.4 The Neural Stem Cell Niche

The stem cell niche regulates stemness or plasticity, specifically, cell maintenance, self-renewal and lineage fate specification (Morrison and Spradling 2008). The microenvironment in which NSCs reside is also known as the NSC niche with the two neurogenic NSC niche areas in the brain located in the SVZ and SGZ (Alvarez-Buylla and Lim 2004). Multiple extrinsic factors in the NSC niche mediate NSC function, including signalling molecules. In addition, neighbouring mature cells and proteins located in the ECM have important regulatory roles influencing NSC behaviour.

6.4.1 The Extracellular Matrix (ECM) and Cellular Components of the NSC Niche

The ECM is described as the substance between and around cells and is produced by the cells that reside within it, thus it has a highly tissue-specific structure (Hubmacher and Apte 2013). The brain ECM is produced by the cells of the nervous system, specifically, neurons, glial cells and non-neural cells types, and it provides an environment for neural cell development and plasticity (Howell and Gottschall 2012; Soleman et al. 2013). During development, the brain ECM supports the formation of neurons and glial cells as well as cell migration, axonal outgrowth and synapse development while in the adult it mediates brain structure and plasticity, e.g. during or after injury or disease (Howell and Gottschall 2012; Soleman et al. 2013). The brain ECM also has an important role in mediating brain plasticity in response to environmental changes and altered neural activity (Howell and Gottschall 2012; Soleman et al. 2013). Importantly, the brain ECM is also responsible for the formation of perineuronal nets (PNNs), aggregates of the ECM found in particular in the cortex, hippocampus, brain stem and cerebellum, key regulators in synapse structure and function and neuronal activity (Howell and Gottschall 2012; Soleman et al. 2013).

Cellular components of the NSC niche include mature cells, such as astrocytes, ependymal cells, vascular cells and mature neurons. The cell–cell contacts between these cells and NSCs contribute to the regulation of NSC self-renewal and lineage specification. Astrocytes express secreted and membrane-bound factors that regulate proliferation and differentiation of NSCs as well as neurogenesis and synaptic plasticity (Barkho et al. 2006; Parpura et al. 2012). Connections between cells in different compartments such as between neurons and the vasculature are made through astrocyte processes (Barkho et al. 2006; Parpura et al. 2012). Ependymal cells are ciliated cells that line the lateral ventricles and are in close contact with SVZ NSCs (Ramirez-Castillejo et al. 2006; Sawamoto et al. 2006). It is thought that these cells regulate NSC maintenance and differentiation through the secretion of soluble factors (e.g. Pigment-epithelium derived factor, Noggin) which promote NSC

self-renewal and neurogenesis (Lim et al. 2000; Ramirez-Castillejo et al. 2006). Endothelial cells and NSCs secrete vascular endothelial growth factor (VEGF), with endothelial cell-derived VEGF promoting NSC proliferation and maintenance, and NSC-derived VEGF promoting endothelial cell proliferation and angiogenesis (Mackenzie and Ruhrberg 2012). Mature neurons also contribute to the NSC niche through the secretion of neurotransmitters and other factors that predominantly promote neurogenesis (Ma et al. 2009). Combined, neighbouring cells surrounding the NSC niche provide structural and nutritional support to the NSC pool.

6.4.2 ECM Proteins in NSC Regulation

The ECM also forms a physical compartment in which NSCs reside with the heterogeneous properties of the NSC niche ECM regulating the balance between NSC self-renewal or differentiation (Reinhard et al. 2016). Several ECM proteins, including PGs, collagens, laminin and integrins have also been implicated to promote NPC self-renewal in the human SVZ where cell adhesion and cell–ECM interactions are crucial in regulating NSC maintenance (Fietz et al. 2012). In the adult mouse hippocampus, the ECM glycoproteins reelin and tenascin-R have been reported to mediate neurogenesis reinforcing the role of ECM proteins in mediating NSC lineage commitment (Zhao et al. 2007; Xiao et al. 2014). Not only providing a structural environment for cell attachment and cell–matrix interaction, the ECM also sequesters GFs, morphogens and cytokines to the NSC niche providing a permissive environment for self-renewal or differentiation (Faissner and Reinhard 2015). In the larger context, the brain ECM plays a crucial role in brain plasticity and homeostasis allowing for larger functions, including the development of memory and learning (Dityatev et al. 2010).

6.5 HSPG Mediated Signalling in the Neural Niche

The balance of NSC plasticity—self-renewal and lineage differentiation capacity—is crucial for maintenance of the NSC pool as well as for sufficient neurogenesis and gliogenesis. GFs play numerous roles in cellular functions including cell growth, proliferation and maintenance. PGs are ubiquitous throughout the neuronal niche where they influence key functions including axonal guidance (Bloechlinger et al. 2004; Lau and Margolis 2010) and neural differentiation (Oikari et al. 2016). In addition, PG core protein/GF interactions can promote or inhibit cell proliferation dependent on GAG chain type, expression pattern and ligand interactions (Pucci-Minafra et al. 2008).

Heparan sulfate (HS) chains are structurally heterogeneous and avidly bind a diverse range of proteins under physiological conditions (Sarrazin et al. 2011). Cell surface HS chains provide cells with a mechanism to share a wide variety of

extracellular effectors without requiring multiple novel binding proteins (Kraemer 1971a, b; Lander and Selleck 2000) and in this way, they regulate multiple biological functions. Soluble proteins bound by HS chains include growth factors such as fibroblast growth factors (FGFs), epidermal growth factor (EGF), VEGF, PDGF and transforming growth factor-beta (TGF-β); morphogens such as bone morphogenetic protein (BMP), Hedgehog (Hh, mammalian: Shh) and Wingless related integration sites (Wnts; Fig. 6.4); and cytokines and chemokines such as interleukins, chemokine ligand 2 (CCL-2) and tumour necrosis factor-alpha (TNF-α) (Bernfield et al. 1999; Tkachenko et al. 2005; Filmus et al. 2008; Dreyfuss et al. 2009).

Along with ECM components, HS chains are also able to bind various cell surface receptors (e.g. FGF receptor; FGFR; Fig. 6.4) and intracellular and extracellular enzymes (Esko and Selleck 2002; Dreyfuss et al. 2009). This variety of binding options imparts HSPGs with extensive influence over signalling pathways with the specificity of signalling interactions dependant on the length of the HS chain, distribution of saccharide residues (GlcA vs. IdoA) as well as the number of *N*-acetyl and *N*-sulfation groups and the number and position of *O*-sulfated areas along the chain (Esko and Selleck 2002; Dreyfuss et al. 2009).

6.5.1 FGF-2 and EGF

One of the most well-characterised pathways mediated by HSPGs is the FGF signalling pathway, where it has been established that HS and FGF interaction is required for fully activated FGF signalling to occur (Dreyfuss et al. 2009; Fig. 6.4). Through their sulfation specificity, HSPGs can enhance the activity of FGF signalling through increasing the affinity of FGF binding to FGFRs and, for complete activation to occur, via HS chains also binding to the FGFRs (Mansukhani et al. 1992; Ornitz et al. 1992; Elenius and Jalkanen 1994; Harmer 2006; Chan et al. 2015).

In NSCs, FGFs and EGF are required for NSC maintenance (Decimo et al. 2012) with the importance of FGFs in the development of the nervous system well documented (Guillemot and Zimmer 2011). FGFs and EGF bind to their receptor tyrosine kinase (RTK) receptors, resulting in the activation of downstream signalling cascades, such as phosphoinositide 3-kinase (PI3K)/Akt or mitogen-activated protein kinase (MAPK) (Schlessinger 2000), with MAPK activation by FGFs increasing cell proliferation (Iwata and Hevner 2009).

The FGFs, comprised of 22 family members, bind four FGF-specific receptors (FGFR1-4), with this binding largely regulated by cell surface proteins such as the HSPGs (Iwata and Hevner 2009; Li et al. 2016). The influence of FGF-2 and EGF on NSC maintenance and proliferation has been demonstrated in vivo where infusion of these growth factors into the adult mouse SVZ resulted in increased proliferation of SVZ NSCs (Ochi et al. 2016). FGFs are required for the survival and expansion of NSCs with FGF-2 a potent mitogen for NSCs and increasing the cells' responsiveness to EGF, which also promotes NSC/NPC proliferation (Aguirre et al. 2010;

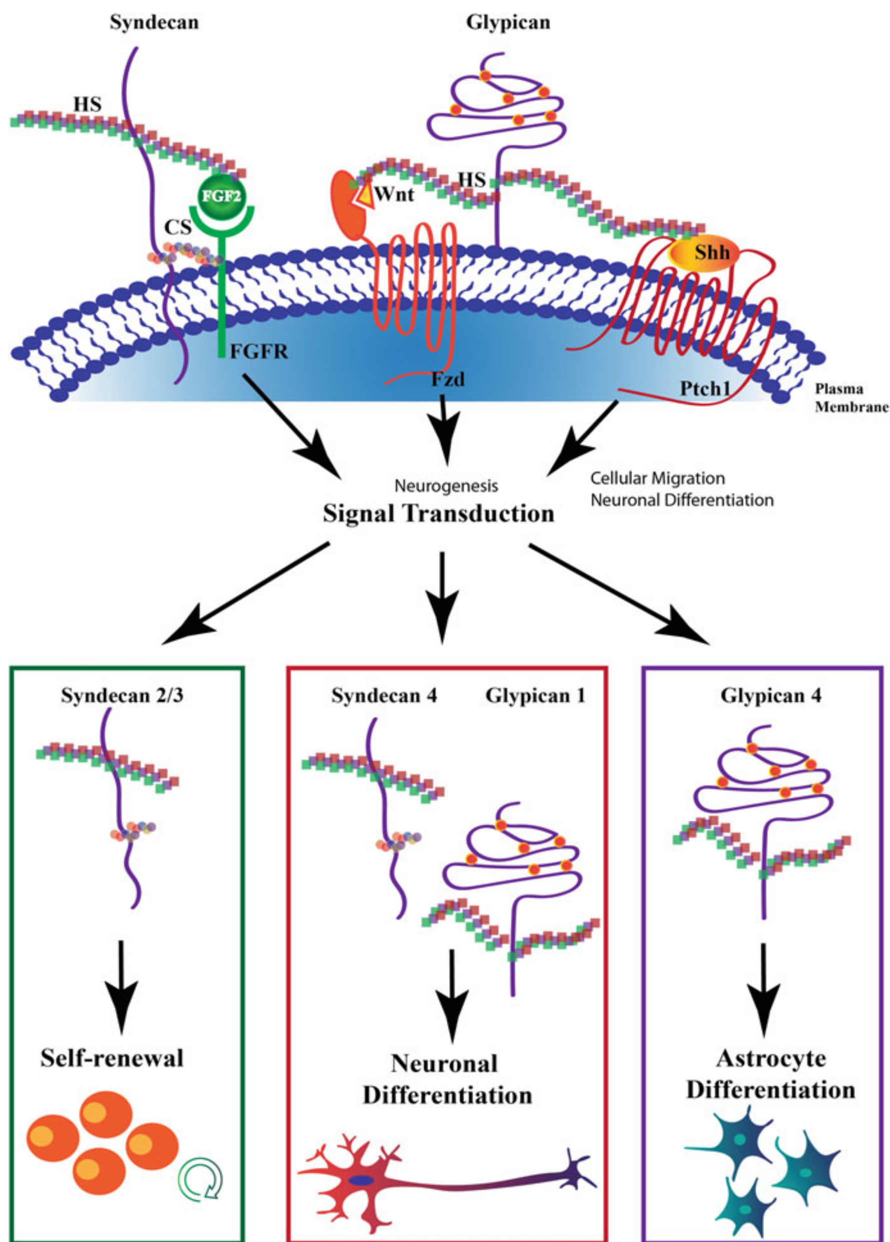

Fig. 6.4 Syndecans and glypicans interact with extracellular molecules and cell surface receptors mediating their downstream signalling. Interactions between syndecans and glypicans and their signalling molecules occur through their heparan sulfate (HS) side chains. Common signalling partners include growth factors (fibroblast growth factor; FGF) and morphogens (Wnt, Shh) along with their receptors (FGFR, Frizzled; Fzd, Patched-1; Ptch1). Specific heparan sulfate proteoglycan (HSPG) core proteins have been associated with specific neural lineages and can be used to drive lineage-specific differentiation (Oikari et al. 2020)

Guillemot and Zimmer 2011). As a result, the combination of FGF-2 and EGF is commonly used for the in vitro culturing of NSCs (Guillemot and Zimmer 2011).

HS chains bound to HSPG core proteins, including SDCs, can contain both FGF-2 binding sites, and sequences inhibitory to the binding of the GF (Guimond et al. 1993). While not the sole signalling partner, FGF signalling is predominantly mediated by the syndecan family. With SDCs expressed by all adhesive cells, these HSPGs augment FGF action in vivo (Elenius and Jalkanen 1994), where normal cell growth is associated with SDC1 upregulation and abrogation of this HSPG suppressing proliferation (Elenius and Jalkanen 1994).

HSPG core proteins may carry binding sites for multiple GFs dependent on GF availability during different developmental stages (Nurcombe et al. 1993). In the case of FGF binding, this may allow a core protein to carry binding sites for multiple FGF molecules (e.g. FGF2, FGF8, etc.). Variable glycosylation patterns on cell surface PGs is dependant on cell type and extracellular factors (Sanderson and Bernfield 1988; Rapraeger 1989; Tkachenko et al. 2005), and minor differences in disaccharide sequences have demonstrated remarkable changes in ligand specificity (Guimond et al. 1993; Nurcombe et al. 1993; Sanderson et al. 1994). This highlights that specific structures of HS are needed for individual receptor and GF complex formation (Guimond et al. 1993).

While much of the effect of HS cell surface molecules on GF-regulated growth control occurs at the cell surface, it is possible for events to occur at the peri-cellular/extracellular level or within intracellular compartments allowing these proteins to exhibit functions of matrix-bound HSPGs such as FGF reservoir formation (Gonzalez et al. 1990; Klagsbrun 1990), control of FGF diffusion (Flaumenhaft et al. 1990) or protection of FGF-2 from proteolytic activation (Saksela et al. 1988; Elenius and Jalkanen 1994).

6.5.2 Wnt/β-Catenin Pathway

The Wnt/β-catenin pathway, also known as the canonical Wnt pathway, mediates NSC proliferation as well as promoting neurogenesis (Ikeya et al. 1997; Lie et al. 2005). In the canonical Wnt pathway, signalling occurs through phosphorylation of intracellular β-catenin leading to the degradation of β-catenin and repression of Wnt target genes (Chen et al. 2000). In contrast, non-canonical signalling occurs when extracellular Wnt binds its receptor Frizzled leading to activation of downstream molecules resulting in the accumulation of β-catenin and transcription of Wnt target genes (Chen et al. 2000). In the adult rat, hippocampus-enhanced Wnt signalling has been shown to increase neurogenesis while inhibition of this pathway leads to decreased neurogenesis (Lie et al. 2005).

The Wnt proteins are cysteine-rich secreted glyco-lipoproteins that regulate development, cell proliferation, motility, cell fate and cell polarity (Lee et al. 2006). Wnts and their downstream signalling pathways have been extensively examined and demonstrated in the self-renewal, differentiation and neurogenesis

of human Mesenchymal Stem Cells (MSCs; Ling et al. 2009). Wnt pathway activity is regulated by intracellular signalling components as well as extracellular factors, specifically the HSPGs where, through variation in binding affinity, HS fine-tunes the access of Wnts to their receptors (Frizzled) regulating several developmental processes (Perrimon and Bernfield 2000).

Depending on the context, GPCs might have a stimulatory or inhibitory activity (Filmus et al. 2008). Most in vivo evidence indicates the main function of membrane-attached GPCs is the regulation of Wnt, Hh, FGF and BMP signalling (Ohkawara et al. 2003; Song et al. 2005). The regulatory activity of GPCs in these signalling pathways is only partially dependent on HS chains (Song et al. 2005; Kirkpatrick et al. 2006; Capurro et al. 2008; Filmus et al. 2008). GPCs can also be shed into the extracellular environment to mediate morphogen gradient formation (Fujise et al. 2003; Belenkaya et al. 2004; Han et al. 2004, 2005; Kirkpatrick et al. 2004; Akiyama et al. 2008). In the case of Wnt, it has been proposed that the stimulatory mechanism is based on the ability of GPCs to facilitate and/or stabilise the interaction of Wnts with their signalling receptors, the Frizzled proteins (Filmus et al. 2008).

HS chains appear essential for some, but not all, GPC activity (Song et al. 1997). In human cells in vitro*,* different GPC core proteins have been found to have opposing effects on Wnt signalling, with GPC3 stimulating and GPC6 inhibiting this pathway (Capurro et al. 2014). In addition, for some core proteins, attachment to, or cleavage from the membrane can have opposing effects on signalling. For example, membrane-bound GPC4 activates both β-catenin dependent and independent Wnt pathways while cleaved GPC4 has been shown to have an inhibitory effect on Wnt signalling (Sakane et al. 2012).

6.5.3 Sonic Hedgehog (Shh) Pathway

Sonic hedgehog (Shh) is one of three mammalian members of the hedgehog (Hh) family of signalling molecules central to regulating many developmental processes, including the formation of the nervous system (Ruiz i Altaba et al. 2002; De Luca et al. 2016). Shh is a soluble molecule that binds and forms a complex with its receptor Patched (Ptc) and the co-receptor Smoothened (Smo). In the absence of Shh, Ptc represses signal transduction from Smo resulting in inhibition of Shh target genes with binding of Shh releasing this inhibition and leading to the activation of Shh target genes (Ruiz i Altaba et al. 2002; De Luca et al. 2016). Shh signalling has also been found to be central to the regulation of cellular migration in the adult mammalian brain (Balordi and Fishell 2007). Together with Wnt signalling, activation of the Shh pathway has been shown to induce dopaminergic neuron differentiation of human pluripotent stem cells (hPSCs) (Kriks et al. 2011) with an increasing body of evidence highlighting the importance of Shh signalling in neuronal differentiation.

Multiple roles for HSPGs in regulating morphogen signalling have also been reported predominantly based on studies in the model organisms *Drosophila melanogaster*, *Xenopus laevis*, zebrafish and rodents (Hacker et al. 2005). Building on studies in *Drosophila* where the homologue was required for Hh signal transduction (Desbordes and Sanson 2003), it has been demonstrated that mammalian GPC3 has a high affinity for Shh (mammalian) and Hh (*Drosophila*), but not for the receptor Ptc, and thus competes with Ptc for Hh binding (Capurro et al. 2008). The binding of GPC3 to Hh results in endocytosis of the GPC3/Hh complex, and the inhibition of Hh signalling (Capurro et al. 2008). Interestingly, this effect of GPC3 on Hh signalling is not completely due to GPC3 HS chains with the GPC3 core protein also binding Hh with high affinity (Capurro et al. 2008).

In contrast to GPC3, GPC5 has been shown to induce Hh signalling through increasing binding of Shh to Ptc instead of competing with the receptor (Li et al. 2011). In addition, GPC5 binds to Hh and Ptc via its HS GAG chains (Li et al. 2011). Finally, GPC1 has been linked to Shh-mediated axon guidance of commissural neurons (Wilson and Stoeckli 2013), highlighting the importance of HSPGs and glypicans in particular, in development and neurogenesis.

6.5.4 Other Signalling Pathways

Multiple other signalling molecules, including TGF-β, PDGF, insulin-like growth factor and members of the hepatocyte growth factor family have been reported to bind to extracellular proteins or HS chains (Taipale and Keski-Oja 1997).

The neurotrophin GFs include brain-derived neurotrophic factor (BDNF), reported to bind HS (Kanato et al. 2009; Huynh et al. 2019), regulates survival, differentiation and migration of neurons in the CNS (Islam et al. 2009; Wei et al. 2015). Neurotrophins bind specific tropomyosin-related kinase (Trk) receptors with BDNF binding to the TrkB receptor (Islam et al. 2009). Ligand binding triggers dimerisation and autophosphorylation of the Trk receptor resulting in activation of downstream signalling cascades and regulation of neuronal cell functions including survival, proliferation and differentiation (Islam et al. 2009). BDNF has been extensively studied and shown to promote NPC survival and proliferation resulting in larger neurosphere diameter in vitro and importantly, to promote neuronal differentiation (Li et al. 2009; Wei et al. 2015).

6.5.5 Platelet-Derived Growth Factor (PDGF)

The platelet-derived growth factor (PDGF) family consists of PDGF-A, -B, -C and -D isoforms that can act as homo- or heterodimers (AA, BB, AB, CC or DD) to bind to the PDGF receptors (PDGFRs) (Shim et al. 2010). The two PDGFR types, PDGFRα and PDGFRβ, can dimerise in the -αα, -ββ or -αβ forms. Similar to

FGFR, PDGFR belongs to the RTK family and upon ligand binding can activate the MAPK and PI3K pathways (Shim et al. 2010; Li et al. 2015). In the CNS, expression of PDGFRα by oligodendrocyte progenitor cells (OPCs) suggests that PDGF signalling is important in driving oligodendrocyte production (Rakic and Zecevic 2003; Jackson et al. 2006). The PDGFRα is expressed by PDGF-responsive neural precursors (PRPs), another subset of cells within the CNS with multilineage differentiation potential which represent a subpopulation distinct from GFAP expressing NSCs (Chojnacki and Weiss 2004; Chojnacki et al. 2011). PDGF, along with FGF-2 and EGF have been used to induce neuronal differentiation of hMSCs (Tao et al. 2005) and PDGF is also an HS-binding protein (Smith et al. 2009).

6.6 HSPGs in Neural Development

Mammalian nervous system development involves the generation of neurons from NSCs, migration of generated neurons towards genetically determined locations, extension of axons and dendrites and establishment of neural connectivity. There are roles for HSPGs in all of these diverse processes (Yamaguchi 2001; reviewed in Matsumoto-Miyai et al. 2009; Dityatev et al. 2010).

6.6.1 SDCs and Neurogenesis

Studies in murine models have demonstrated that all four SDCs are expressed in the mammalian brain, each with their own specific expression pattern during embryonic development as well as in the adult CNS (Hsueh and Sheng 1999; Ford-Perriss et al. 2003). SDCs are expressed in developing brains, including dendritic spine morphogenesis and developing axon tracts (reviewed in Yamaguchi 2001).

Distinct differences in SDC1 expression have been observed between the developing and adult mouse brain associated with differences in cellular proliferation as well as distinct regional expression differences (Hsueh and Sheng 1999; Ford-Perriss et al. 2003). In vivo and in vitro studies have further demonstrated SDC1 in the maintenance and proliferation of mouse NPCs through a Wnt-pathway mediated mechanism (Wang et al. 2012).

Distinct patterns of expression for other SDC core proteins have been observed in the developing mouse brain with the expression of some proteins (SDC2, SDC3) restricted almost exclusively to neurons (Ford-Perriss et al. 2003). In the rodent brain, SDC2 is localised to synapses during brain maturation and appears to be required for dendritic maturation of hippocampal neurons where it is expressed during dendritic spine morphogenesis (Ethell and Yamaguchi 1999; Hsueh and Sheng 1999; Yamaguchi 2001; Ford-Perriss et al. 2003). In contrast, SDC3 expression in the adult rat brain is concentrated to specific cells and regions (Hsueh and Sheng 1999). Due to its strong expression in developing rodent axon tracts

(reviewed in Yamaguchi 2001), SDC3 has been implicated in neural cell migration (Hsueh and Sheng 1999; Hienola et al. 2006) perhaps through its function as a receptor for glial-derived neurotrophic factor (GDNF), responsible for the stimulation of neurite outgrowth and neuronal migration (Bespalov et al. 2011). In the adult rodent brain, SDC4 is expressed in white matter, demonstrating glial-specific expression, and its involvement in glial function is further supported through mediation of rat astrocyte adhesion (Hsueh and Sheng 1999; Avalos et al. 2009).

Less evidence exists in the human; however, SDC2 and SDC3 (*N*-syndecan) appear to contribute to synaptic plasticity and synapse maturation (Leonova and Galzitskaia 2013). SDC2 is localised specifically to synaptic junctions (Hsueh et al. 1998) where interactions between SDC2 and postsynaptic density protein 95/Discs large protein/Zonula occludens 1 (PDZ; Shimada et al. 2019) family proteins (those carrying a PDZ binding motif) are required for the development of postsynaptic specialisation (Oohira et al. 2000).

6.6.2 GPCs and Neurogenesis

HSPG GPCs also demonstrate specific expression patterns in the mammalian CNS and are implicated in regulating neural cell functions. In the developing rat brain, GPC1 is expressed in areas of proliferating precursor cells, while in the adult GPC2 expression localises to mature neurons, and specifically their axons (Litwack et al. 1998). GPC1 is thought to regulate axonal guidance and branching as well as neural cell migration (Ronca et al. 2001) with GPC1 KO mice identified to develop a small brain phenotype, suggested to be due to a transient reduction in FGF signaling (Jen et al. 2009).

GPC2, predominantly neuronal and specifically expressed in mitotic neurons in the developing mouse brain (Stipp et al. 1994; Ford-Perriss et al. 2003), localises to actively growing axons where it exerts its regulatory role in neuron function by promoting neurite outgrowth and cell adhesion (Kurosawa et al. 2001). Limited data exists for the role of GPC3 in nervous system function despite specific regional expression in the developing mouse brain (Luxardi et al. 2007).

GPC4 is highly expressed in the nervous system with multiple functions in regulating the nervous system and neural cell function. Areas of GPC4 expression in the mouse brain correlate with areas containing NSC pools in both the developing brain and the adult (Hagihara et al. 2000). This suggests a role for GPC4 in regulating NSC maintenance and function (Hagihara et al. 2000). In the adult, GPC4 expression has been demonstrated in the DG (Hagihara et al. 2000), one of the few sites where neural stem cells are detected to continually replicate in adult rat models (Altman and Das 1965; Yamaguchi 2001). In culture, NSCs have been shown to express GPC4 on their cell surface with this expression restricted to both mouse ESCs and NSCs (Hagihara et al. 2000; Fico et al. 2012) and to be required for self-renewal and maintenance with expression of GPC4 ceasing once cells commit to neural differentiation (Hagihara et al. 2000). In addition to functions maintaining

self-renewal, GPC4 has been implicated in synapse function and is required for excitatory synaptic transmission in neurons (Ko et al. 2015). In combination with GPC6, GPC4 has also been shown to be secreted by astrocytes and is required for postsynaptic glutamate receptor clustering, with defects in synapse formation evident in GPC4-null mice (Allen et al. 2012).

Early in foetal development, GPC5 expression is localised to postmitotic neurons but has also been detected in adult mammalian brains (Saunders et al. 1997; Luxardi et al. 2007). In nervous system auto-immune diseases such as the demyelinating diseases multiple sclerosis and neuromyelitis optica, GPC5 polymorphisms have been implicated in an Asian population (Shin et al. 2013). Enriched expression of GPC6 has been detected in the mouse cerebellum (Allen et al. 2012) and GPC6 has been found to regulate FGF-2 signalling during mouse cerebral cortical development (Salehi 2009).

6.6.3 Matrix Localised Perlecan

During mammalian neural development, the HSPG perlecan is expressed in the basal lamina of the neuroepithelium, a crucial component of the neural niche (Ford-Perriss et al. 2003). Perlecan is a multi-domain HSPG of the ECM with diverse roles during development and organogenesis (Knox and Whitelock 2006). A large protein with five domains, perlecan carries 3–4 HS chains in domain I and one in domain V (Fig. 6.1), which interact with heparin-binding growth factors such as FGF-2, EGF, and VEGF (Farach-Carson and Carson 2007). Perlecan is another multifunctional protein in the developing mouse brain, where it promotes NSPC proliferation in the SVZ by acting as a co-receptor for FGF2 (Nurcombe et al. 1993; Yamaguchi 2001; Giros et al. 2007). FGF2 is essential in the mouse NSC niche and requires perlecan to promote proliferation through activation of the Akt and Erk1/2 pathway, elegantly demonstrated by Kerever et al. (2014) in a perlecan-null murine model. In the same study, FGF2 failed to promote neurosphere formation of GFAP^{+}CD133^{+} NSCs owing to the inability to induce cell cycle progression via cyclin D2 (Kerever et al. 2014). In another study by Girós et al. (2007), perlecan-null mice displayed impaired forebrain development, through disrupted Shh signalling, with perlecan essential for mediation of the Shh concentration gradient (Giros et al. 2007; Palma et al. 2011).

In vitro studies by Nakamura et al. (2015) demonstrated the addition of exogenous perlecan to neural cultures promoted NSPC proliferation as well as neurite extension. The addition of heparin (a highly sulfated short HS) was found to promote NSPC proliferation, although this was not significant, suggesting perlecan as well as its associated GAGs are required. Perlecan has also been demonstrated to play a role in gliogenesis, where OPCs show increasing expression of perlecan during terminal differentiation of mature oligodendrocytes (Winkler et al. 2002). In contrast, perlecan expression in astrocytes has been shown to inhibit proliferation due to domain V, with heparin also shown to a lesser extent to suppress astrocyte proliferation (Nakamura et al. 2015). With its contribution to differentiation of the astrocyte

and oligodendrocyte lineages, perlecan is likely a marker for glial lineage specification.

6.7 Stem Cell Classification

Stem cells are defined as undifferentiated cells with the capacity to self-renew and differentiate into specialised cell types (Fuchs and Chen 2013). Embryonic stem cells (ESCs) are derived from the inner cell mass of the developing blastocyst and are pluripotent, capable of differentiating into any of the three embryonic layers, the endoderm, mesoderm or ectoderm, thus creating all tissues in the body (Thomson et al. 1998; Trounson 2006). ESCs are capable of unlimited proliferation in vitro in an undifferentiated state, reflected by high telomerase expression when detected using a Telomeric Repeat Amplification Protocol - assay (Thomson et al. 1998).

Adult stem cells are multipotent with limited differentiation capacity. They reside in specific tissues where they generate tissue-specific cells, thus being responsible for the regeneration of various tissues including but not limited to skin and neural tissue (Calvi and Link 2015; Gregoire et al. 2015; Ojeh et al. 2015). Generally, stem cells give rise to progenitor cells, with restricted proliferation, self-renewal and differentiation capacity, before undergoing further specialisation producing cells of a single lineage (Seaberg and van der Kooy 2003; Fuchs and Chen 2013; Homem et al. 2015). These cells are primarily distinguished from each other by their ability, or lack of, life-long self-renewal (Fuchs and Chen 2013).

6.7.1 Neural Stem Cell Characterisation

NSCs are multipotent stem cells of the CNS that have the ability through asymmetric division to generate neurons and glial cells, including astrocytes and oligodendrocytes (Gage 2000). NSCs are characterised by the expression of specific markers that determine intracellular, cell surface and behavioural characteristics. Commonly used intracellular markers for the identification of NSCs include nestin (intermediate filament), vimentin, SRY-box 1 and 2 (SOX1 and SOX2; high mobility group (HMG)-box transcription factors) and Musashi 1 (MSI1; RNA-binding protein). These markers mediate self-renewal thus contributing to the maintenance of the NSC pool (Ellis et al. 2004; Okano et al. 2005; Park et al. 2010; Christie et al. 2013; Ramasamy et al. 2013). NSCs also exhibit astrocytic characteristics and have been shown to express the astrocyte marker GFAP (Codega et al. 2014). Some markers such as CD133 are used for the isolation of NSCs (Corti et al. 2007) while markers such as bromodeoxyuridine (BrdU) and Ki67 are used to determine the quiescent and proliferative state of NSCs (Rao et al. 2006; Decimo et al. 2012). The self-renewal and multilineage differentiation potential of NSCs also needs to be monitored when characterising NSCs (Ramasamy et al. 2013).

6.7.2 Neural Stem Cell Expansion and Derivation

Following the discovery in the rodent that neurogenesis persists in the adult mammalian brain (Reynolds and Weiss 1992; Palmer et al. 1995), identification and isolation of human NSCs was achieved in the late 1990s (Kukekov et al. 1999). This led to the establishment of rodent and human NSC in vitro isolation and culturing protocols with multiple rodent and human NSC lines now routinely expanded and commercially available. The culture conditions for the in vitro propagation of both rodent and human NSCs are similar, using serum-free conditions along with the presence of key GFs, EGF and FGF2, to maintain cells in an undifferentiated and multipotential state (Sun et al. 2008). The two main methods of expanding NSCs in culture are the neurosphere assay and the adherent monolayer culturing system.

6.7.3 Neurosphere Assay

The neurosphere assay allows the identification, isolation and in vitro propagation of NSCs (Reynolds and Weiss 1992). The generation of free-floating clusters of cells comprised of NSCs, progenitor and differentiated cells occurs in serum-free culture in the presence of EGF and FGF2. During sphere formation, extraneous cells die leaving only culture condition responsive NSCs (Ramasamy et al. 2013). Primary neurospheres generated through this process can then be passaged to secondary spheres allowing further propagation or differentiation towards neural lineages (Reynolds and Rietze 2005). The ability to form secondary spheres and their subsequent ability to differentiate represents the self-renewal and multipotent differentiation capacity of NSCs (Reynolds and Rietze 2005). This culture and propagation of NSC neurospheres is challenged by high cell heterogeneity and the low number of NSCs within a neurosphere (Conti et al. 2005).

6.7.4 Adherent Monolayer

In adherent long-term culturing, the presence of both EGF and FGF2 is required to support symmetrical division of NSCs and their multilineage differentiation potential (Conti et al. 2005). Protocols for the stable adherent culture of rodent and human NSCs are now well established with differentiation of adherent NSC cultures achieved through plating of the cells on ECM substances such as laminin (Conti et al. 2005). An advantage of these methods includes maintaining high levels of homogeneity, reduced spontaneous differentiation and the potential to expand the cells over 40 passages or approximately 100 generations (Sun et al. 2008).

6.8 Models of Human Neurogenesis

6.8.1 Embryonic Stem Cell-Derived NSCs

Pluripotent ESCs have a high expansive potential, thus ESC-derived NSCs provide an advantageous additional mechanism to generate NSCs (Zhang et al. 2001). In defined culture-conditions, it is possible to generate tissue-restricted stem cells from ESCs and in appropriate conditions, ESCs readily acquire a neural identity by default (Tropepe et al. 2001).

Several protocols for the derivation of NSCs from ESCs have been described, including the generation of embryoid bodies (EB) which, in the presence of FGF2, differentiate into neural precursor cells (Zhang et al. 2001; Okano et al. 2005). Other methods result in enriching the cell population for neural precursors that can differentiate into the three neural cell types (Pera et al. 2004; Itsykson et al. 2005), or involve direct differentiation of adherent cells in the presence of GFs, morphogens or their inhibitors (Ying et al. 2003; Shin et al. 2006).

The transcriptome of human ESC-derived NSCs has been found to closely resemble that of primary human NPCs isolated from human foetal brain tissue (Stein et al. 2014). In addition, when differentiated for the same period of time, human ESC-derived NSCs and NPCs maintained a high level of similarity (Stein et al. 2014). However, while many of the same genes are expressed between ESC-derived NSCs and primary NPCs, differences are apparent, including the expression level of signalling pathway receptors and downstream molecules (Shin et al. 2007).

6.8.2 Induced Pluripotent Stem Cell (iPSC) Derived NSCs

NSCs can be derived from induced pluripotent stem cells (iPSCs). iPSCs were first generated by Takahashi and Yamanaka in 2006 when they reported that fibroblasts can be reprogrammed into pluripotent stem cells through the introduction of defined factors, including octamer-binding transcription factor 3/4 (Oct3/4), SOX2, c-Myc and Kfl4 (Takahashi and Yamanaka 2006). Since then, techniques for the derivation of NSCs or neural cells from iPSCs have been described and their potential as a tool of cell therapy for CNS regenerative applications has been demonstrated (Nizzardo et al. 2010; Lopez-Serrano et al. 2016). The advantage of using iPSCs is that they can be generated from easy-to-access cells, such as fibroblasts, and due to their pluripotency, they have the ability to generate all neuronal cell lineages (Nizzardo et al. 2010; Lopez-Serrano et al. 2016). In 2008, Dimos et al. generated iPSCs from a patient with amyotrophic lateral sclerosis (ALS) and successfully differentiated these cells to motor neurons, which are destroyed in ALS (Dimos et al. 2008). However, as oncogenic genes are targeted to achieve cell reprogramming, more research is required for the use of iPSC-derived NSCs for therapeutic applications to ensure

the safety of treatments to recipients (Dimos et al. 2008; Lopez-Serrano et al. 2016) (Fig. 6.5).

6.9 Human Mesenchymal Stem Cells

The term mesenchyme describes the embryonic loose connective tissue that is derived from the mesoderm and that develops into haematopoietic and connective tissues (Lindner et al. 2010). MSCs, like most adult stem cells, are multipotent and can produce a number of cell types from a limited number of population doublings. Undifferentiated MSCs express neuronal and glial markers and due to this, they have generated interest as a therapeutic alternative to NSCs (Foudah et al. 2013). The multipotentiality of hMSCs, along with their easy isolation and expansion in vitro, and their high ex vivo expansive potential make these cells an attractive therapeutic tool in a wide range of clinical applications (Minguell et al. 2001). However, expression of neural markers in MSCs, as observed following differentiation, does not indicate that MSCs are functional neurons and this is the focus of numerous current studies (Foudah et al. 2013; Okolicsanyi et al. 2018).

6.9.1 Isolation

Human mesenchymal stem cells (hMSCs) are generally derived from the bone marrow (Mackay et al. 1998; Pittenger et al. 1999) and adipose tissue, and have the ability to differentiate to cells of mesodermal, endodermal and ectodermal origin such as fat, cartilage, bone and neurons (Alexanian 2010; Okolicsanyi et al. 2015). These cells can also be sourced from other tissues including umbilical cord blood, adipose tissue (Cunningham et al. 2006), salivary glands (Rotter et al. 2008) and from organs such as the gut (Lanzoni et al. 2009). As MSCs are derived from different sources, their viability, yield and differentiation potential vary, with increasing evidence that the source as well as a method of isolation influences their expansive potential (reviewed in Hass et al. 2011; Ragni et al. 2013). Various methods exist to isolate MSCs from the bone marrow (Prockop 1997; Conget and Minguell 1999) resulting in a homogeneous population of plastic-adherent fibroblast-like cells considered the primary ex vivo source of MSCs (Friedenstein et al. 1976; Castro-Malaspina et al. 1980; Minguell et al. 2001).

6.9.2 Characterisation

Currently, MSCs are still identified and characterised by a combination of in vitro morphological, immune phenotypical and differentiative characteristics including

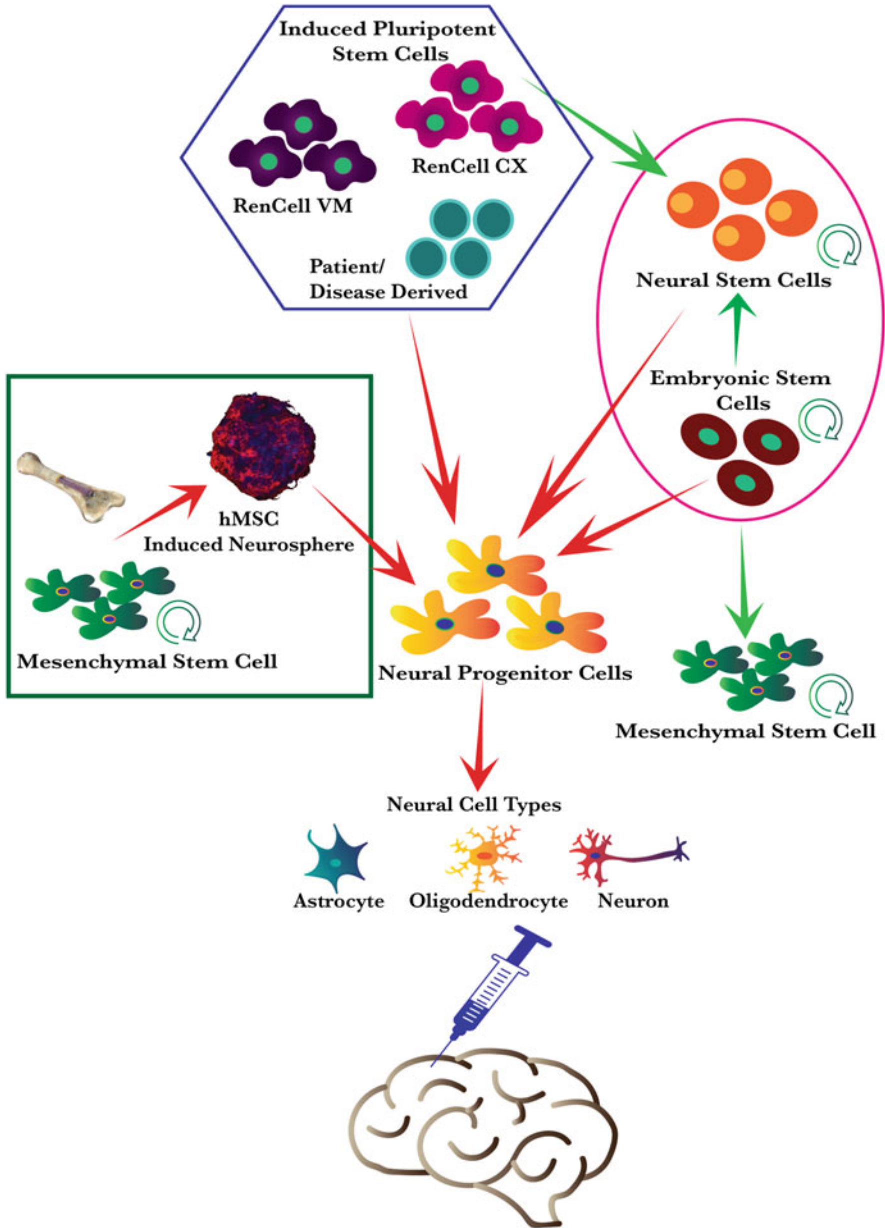

Fig. 6.5 Schematic showing the models used to produce neural cells for therapeutic applications. Induced pluripotent stem cells, neural stem cells and mesenchymal stem cells have all been differentiated to produce neural progenitor cells which can then be differentiated into all three neural cell types, astrocytes, oligodendrocytes and neurons. Generating sufficient numbers of these terminal cell types and being able to control population proportions will be important for end point therapeutic applications

lineage capacity (Koopman et al. 1993; da Silva Meirelles et al. 2008; Lindner et al. 2010). These marker combinations define their plasticity or their multilineage potential with cell surface markers analysed by both flow cytometry and immunocytochemistry commonly used to confirm these features. In addition to their ability to differentiate down the traditional tri-lineages (bone, cartilage, fat), MSC populations should be >95% positive for some combination of CD29, CD44, CD73, CD90 and CD105 and <2% positive for CD11b, CD14, CD19, CD34, CD45, CD79α and HLA-DR (Sadan et al. 2009; Lindner et al. 2010).

6.9.3 Expansion

MSCs have an impressive capacity for in vitro expansion, with some populations continuing to undergo population doublings (PD) past passage +20 (P+20; >70 PD; Okolicsanyi et al. 2014). While this is not true for all populations, it does allow significant in vitro expansive potential for therapeutic applications. While many studies are still conducted at low P+, more recent studies suggest the cells themselves have a far greater capacity for self-renewal prior to entering senescence than previously thought. In addition, continued expression of mesenchymal and neural markers throughout extended in vitro expansion suggests maintained neural potential (Okolicsanyi et al. 2015) (Fig. 6.6).

6.9.4 Differentiation

The expression of neural-specific markers has been observed in cells derived from adult bone marrow in both rodents (Brazelton et al. 2000) and humans (Hermann et al. 2004; Hermann et al. 2006; Alexanian 2010; Fox et al. 2010; Okolicsanyi et al. 2015). Pittenger et al. (1999) concluded that basal nutrients, cell density, spatial organisation, mechanical forces, GFs and cytokines have a profound influence on MSC differentiation (Pittenger et al. 1999). A number of methods have been developed to produce functional neural cells including simultaneous treatment of hMSCs with inhibitors of DNA methylation and histone deacetylation as well as pharmacological agents to increase intracellular cAMP (Alexanian 2010) or the conversion to neuroprogenitor-like spheroids (induced neurospheres) in the presence of EGF/FGF2 (Hermann et al. 2004; Okolicsanyi et al. 2018).

Successful differentiation to neural-like cells has been demonstrated via the detection of neural markers, through immunocytochemistry and gene expression, as well as the secretion of neurotrophic factors (BDNF, GDNF) required for the survival and growth of neuronal cells (Xiong et al. 2014). In addition, the intermediate sphere-like structures produced during differentiation express high levels of early neuroectodermal (NeuroD1, Neurog2, MSI1) markers as well as the loss of expression of mesodermal stromal cell markers (fibronectin) (Hermann et al. 2004).

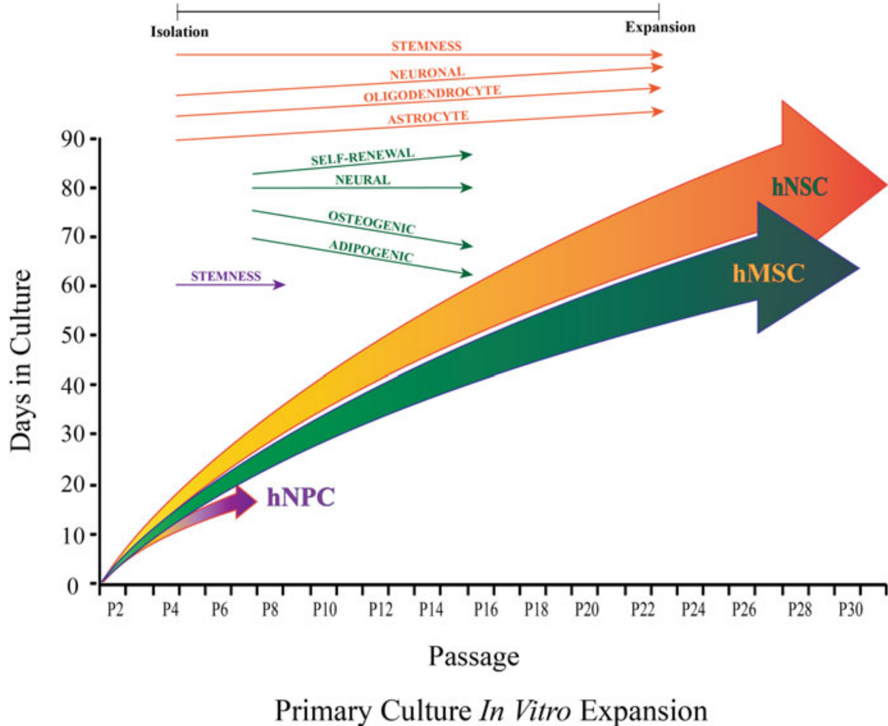

Fig. 6.6 Primary culture in vitro expansion. Human mesenchymal stem cell (hMSC) and human neural stem cell (hNSC) cultures have been successfully expanded in vitro past P+31, >80 days in culture. Human neural progenitor cells (hNPCs) have been expanded in vitro to P+5, ~20 days in culture. hNSC cultures maintain stemness marker expression throughout expansion and demonstrate increased neural lineage marker expression. hMSCs also maintain expression of neural markers throughout the expansion and demonstrate decreased expression of mesenchymal lineage-specific markers and minor increases in neural self-renewal markers. Stemness marker expression is maintained throughout the expansion of hNPC cultures (adapted from Okolicsanyi et al. 2018; Oikari et al. 2016)

These NSC-like cells differentiated into the three neural lineages (astroglia, oligodendroglia and neurons) exhibiting both morphological and functional neural cell characteristics (Hermann et al. 2004).

6.9.5 hMSC-Induced Neurosphere Formation Versus Terminal Differentiation

Among the many different published methods of MSC neural differentiation, two major approaches have emerged. The direct terminal differentiation of MSCs to neural cell types is achieved through the addition of various chemical or epigenetic

stimulants (Alexanian 2010; Fox et al. 2010), and/or the induction of MSCs to form neural stem cell-like spheres prior to terminal differentiation (Hermann et al. 2006; Okolicsanyi et al. 2018). While these methods produce cells with neural cell type morphology, along with a gene and protein expression profile characteristic of neural cells, the functional nature of the cells is still suboptimal.

The use of intermediate NSC-like spheres allows differentiation down each of the three neural lineages rather than the restricted differentiation to neurons and astroglia achieved through direct terminal differentiation (Hermann et al. 2006). In the majority of published protocols, this neurosphere induction involves the addition of FGF2 and EGF in combination with high cell density (Hermann et al. 2004; Fox et al. 2010; Okolicsanyi et al. 2018) to produce sufficient spheres for downstream experiments. Further differentiation to the three neural lineages involves subsequent removal of EGF/FGF2 and plating to substrate coated (ECM) culture vessels and the sequential addition of specific GFs, similar to NSC protocols. The GFs most commonly used include BDNF for neural differentiation and PDGF for glial differentiation (Fox et al. 2010; Chen et al. 2013; Funa and Sasahara 2014; Oikari et al. 2020) in combination with agents such as retinoic acid resulting in decreased proneural gene expression (SOX1, OTX1, NeuroD1 and Neurog2) and increased mature neural markers (GFAP, MBP and TH) (Hermann et al. 2004) (Figs. 6.7 and 6.8).

6.10 Where to Next?

6.10.1 Heparan Sulfate PGs and Neural Stem Cells: A Target for Therapy?

Human neurogenesis and neural development are complex processes involving successive steps of cell migration and differentiation as well as associated signalling events (Wilson and Edlund 2001; Choi et al. 2006). An improved understanding of the mechanisms that mediate hNSC self-renewal and lineage-specific differentiation is crucial for the development of therapeutic strategies for utilising NSCs for brain injury repair, but also for our understanding of the processes regulating human neurogenesis.

Early studies in rodent models implicated HSPGs as mediators of NSC and NPC function in vitro (Hagihara et al. 2000; Abaskharoun et al. 2010; Araujo et al. 2010; Pickford et al. 2011; Fico et al. 2012; Wang et al. 2012). While similarities between the human and rodent models exist, these models also exhibit differences in key cellular properties. However, in both humans and rodents, HSPGs, ubiquitous to the nervous system and its microenvironment, regulate multiple cellular behaviours and may provide novel targets to unravel the complex interactions controlling NSC plasticity.

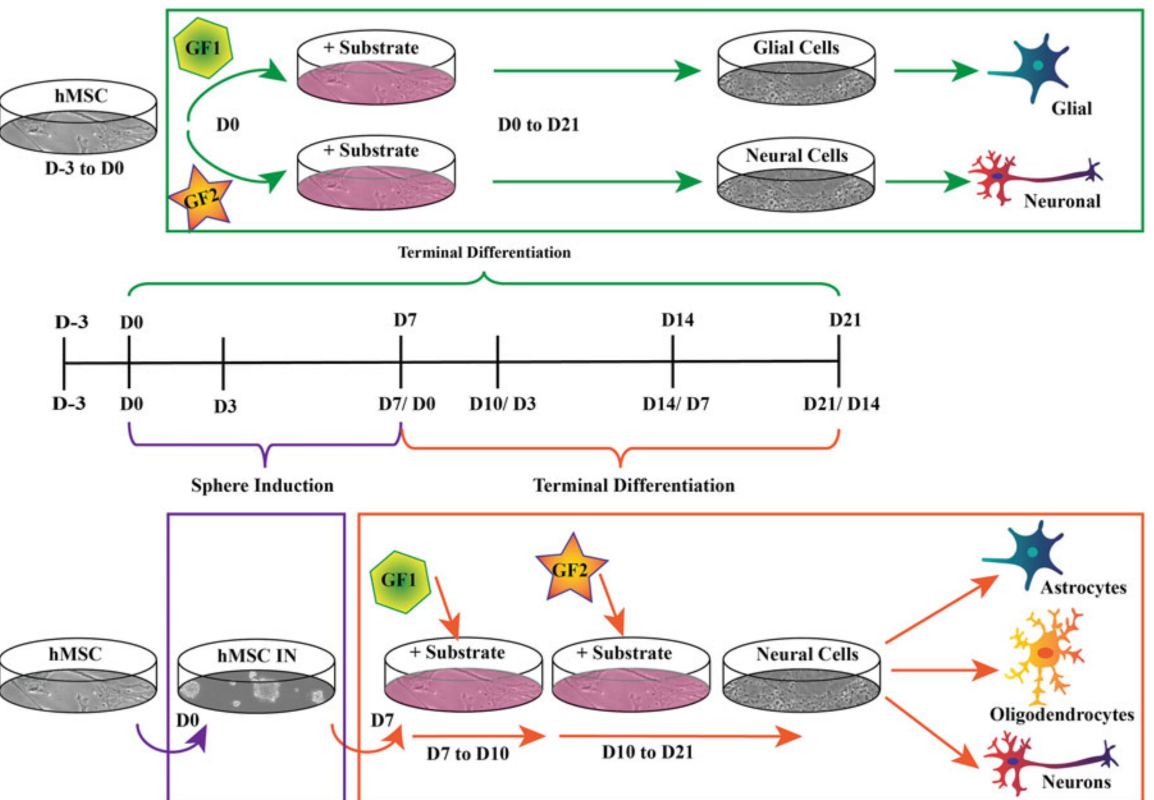

Fig. 6.7 Schematic comparing terminal differentiation and differentiation via induced neurosphere formation. (1) Monolayer cultures are transferred onto a growth substrate where specific growth factors are introduced to the cultures to direct differentiation towards glial or neural lineages. (2) Monolayer cultures are transferred to low-attachment dishes for the induction of neurospheres (IN). Subsequently, IN are transferred onto a growth substrate and undergo sequential addition of specific growth factors to differentiate towards all three neural lineages

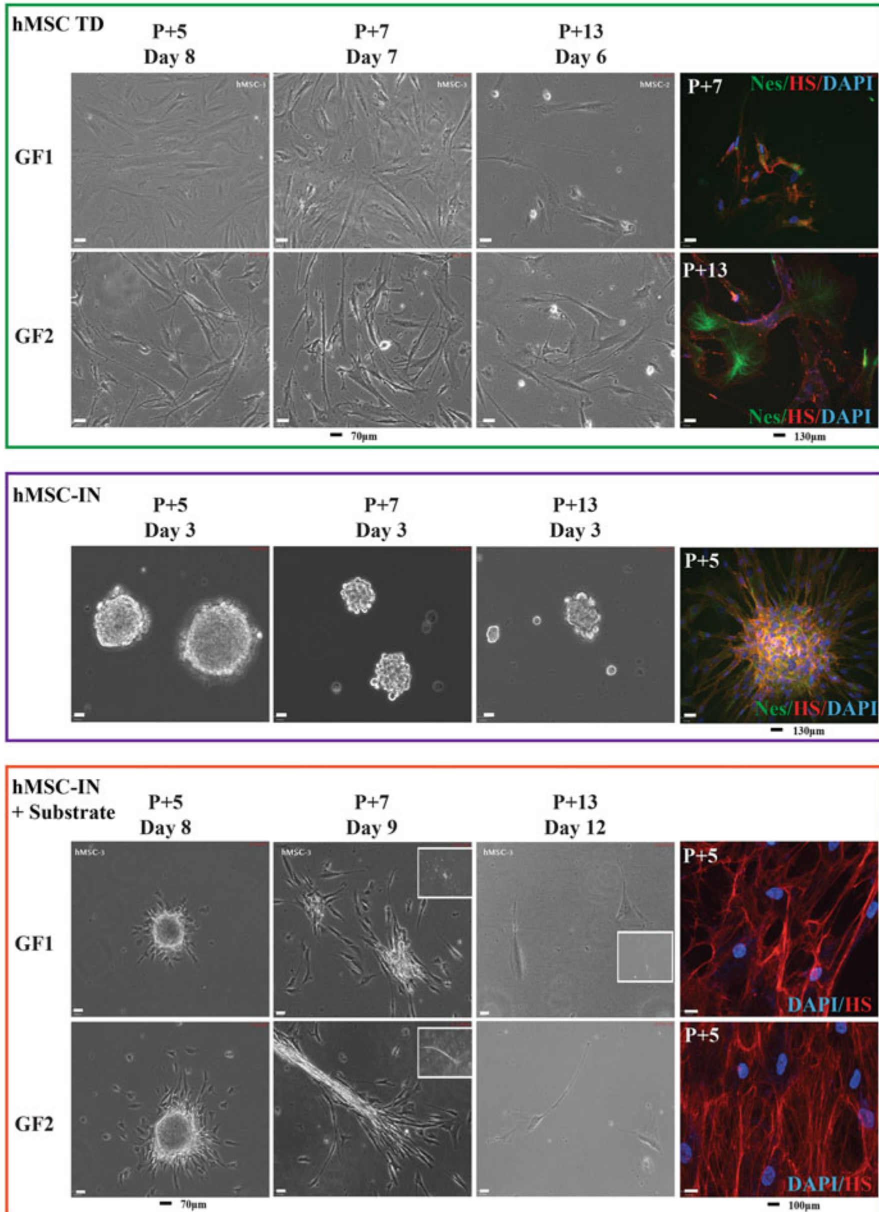

Fig. 6.8 Phase-contrast images of cells undergoing (1) direct terminal differentiation (TD); (2) induced neurosphere (IN) formation; (3) terminal differentiation following IN formation (hMSC-IN + Substrate). ICC images demonstrate the expression of neural markers of self-renewal (Nestin; green) and HSPGs (red). Nuclei are counterstained in blue

6.10.2 Approaches for Exploiting HSPGs as Therapeutic Targets

As key regulators of neural plasticity and lineage specification within the hNSC niche, HSPGs may provide biomarkers for the identification, characterisation, isolation and therapeutic application of hNSCs. This may include exploitation of the cell membrane-associated or ECM localisation of HSPGs within the neural niche, to enhance cell survival and proliferation, neural differentiation or migration of transplanted hNSCs. In addition, the identification of the PGs mediating NSC self-renewal and differentiation will improve the efficacy of human stem cells with neural differentiation potential, including hMSCs and hiPSCs, to be more readily utilised for autologous transplantation. By targeting HSPGs, it is likely we can enhance extracellular signalling efficiency to control lineage specification.

As stem cell fate can be directed through exogenous and synthetic HSPGs and GAGs, exogenous heparin, the highly sulfated analogue of HS, is a widely utilised means of enhancing HS-mediated stem cell proliferation and self-renewal. The study performed by Pickford et al. (2011), identified the heparin effect is dependent on size (length) and concentration (Pickford et al. 2011), and varies between cell culture systems with sensitivity differing between cultures (Mimura et al. 2011). In order to enhance the effect of heparin, a popular strategy is the conjugation of heparin with assorted biomaterials (reviewed in Sakiyama-Elbert 2014). One example is the Sakiyama-Elbert group (2014) who devised an affinity-based delivery system through the combination of a heparin/GF biomaterial. They suggest this conjugation enhances the capability of heparin to bind a wide range of GFs, preventing degradation and potentiating receptor binding via a controlled release mechanism (Willerth et al. 2008).

Harnessing HSPG and growth factor interactions has also resulted in various forms of HS mimetics and synthetic glycopolymers that mimic naturally occurring HSPGs and their function during stem cell neural differentiation. These strategies have produced synthetic glycopolymers with even higher efficacy than heparin. In other studies, Liu et al. (2017) devised a phospholipid-anchored GAG-mimicking polymer, termed “lipo-pSGF”, to promote neural differentiation, likely via the FGF2-Erk1/2 pathway, in mESC cultures and have compared its efficacy to non-anchored pSGF and heparin treated cultures. The lipid-anchor maintained the lipo-pSGF on the cell surface without being exocytosed (Liu et al. 2017). In another variation, Huang et al. (2014) generated synthetic neoproteoglycans (neoPGs) that promoted neural differentiation of mESCs via the FGF2/Erk pathway (Huang et al. 2014). Similarly, Pulsipher et al. (2015) covalently attached GAG derivatives to the cell membrane of mESCs via a HaloTag protein (HTP) anchor, which provided a longer (more than a week), stable presentation of defined HS GAGs when compared to lipid-anchoring (Pulsipher et al. 2015). Through both the HTP anchor and defined sulfation pattern on the HS derivative, the FGF/Erk pathway was activated to promote enhanced exit of mESCs from self-renewal to differentiation of neuronal cell types.

Studies utilising tumour necrosis factor-alpha (TNF-α) have generated cells with an astroglia-like cell morphology while decreasing nestin transcription, suggesting a premature neural phenotype in TNF-α treated hMSCs (Egea et al. 2011). The potential to explore the involvement of TNF-α in neural differentiation is interesting due to the suspected proinflammatory effect of TNF-α in neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease where it is elevated (Hallenbeck 2002; Greig et al. 2004). TNF-α is also reported to have a neuroprotective role. Despite these promising data, there is no current compound that fully mimics natural HSPGs (Pulsipher et al. 2015). However, synthetic GAGs have some advantages over natural GAGs, and provide the opportunity to synthesise compounds of structural homogeneity, purity and controlled sulfation to circumvent current limitations (Wang et al. 2015, 2017) and more importantly to tune cell models to suit desired experimental and thereapeutic endpoints to the repair and recovery of localised damage (Turrin and Rivest 2006).

6.11 Conclusion

Through their complex and variable interactions, HSPGs and PGs, are appropriate targets for exploitation in therapeutic applications. The identification of PGs in numerous diseases including multiple types of cancer and neurological diseases suggests that a single therapeutic target is not feasible. However, multiple candidates warrant further investigation. PGs could be used to drive differentiation and repair through their numerous interactions with the GFs required throughout these processes. Specifically, PGs could be used to simply increase in vitro cell numbers prior to use in therapy for increased therapeutic efficacy or to drive the generation of neural cell populations tailored to specific therapeutic applications.

PG proteins are critical for numerous cellular processes throughout development and retain these critical functions throughout life. As such this protein family forms an inviting target to improve therapeutic options; to control or reverse disease progression or for cellular regeneration for therapeutic applications. With their potential for use as both markers of neural plasticity and lineage specification as well as biomarkers to drive and modulate the stages of neurogenesis, the continued focus on PG core proteins and their biosynthetic machinery will likely yield important advances to our understanding of human neurogenesis as well as for the continued development of therapeutic targets.

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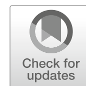
Chapter 7
Syndecan-3: A Signaling Conductor in the Musculoskeletal System



F. K. Jones, O. Kehoe, A. Daroszewska, R. J. van't Hof, and A. Pisconti

Abstract Syndecan-3 is a transmembrane proteoglycan, the largest of the four syndecan family members and the most mysterious. To date, the number of published studies involving syndecan-3 is only a small fraction of the number of studies involving the other subfamily member syndecan-1, yet, these few studies on syndecan-3 cover a whole range of biological functions and phenotypes suggesting that syndecan-3 might be the most eclectic and intereting of the four syndecans. In this review, we focus on what is currently known about the role of syndecan-3 in the musculoskeletal system, from development to aging, including regeneration and disease. We also briefly refer to known syndecan-3 functions in other tissues and systems in an attempt to summarize the most up-to-date knowledge while also instilling curiosity about this extraordinary biomolecule and, hopefully, inspiring more research into its biochemistry and biological functions.

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7.1 Introduction

In vertebrates, the various internal organs of the body and the brain are supported and protected by a complex set of structures collectively known as the musculoskeletal system. In addition to muscles and the bones of the skeleton, which represent the main support structures of the body, the musculoskeletal system also contains other anatomical parts that connect bones and muscles, allowing them to work together in a coordinated way. These are the joints, ligaments, and tendons.

Although a key function of the musculoskeletal system is to provide locomotion, several other functions are carried out by its various components as well. For example, bones participate in the regulation of many biological processes, including metabolic regulation. The mineralized skeleton provides the largest reservoir of minerals in the body. Moreover, through their endocrine function, bones regulate calcium and phosphate homeostasis, mainly via secretion of FGF23 and osteocalcin, and bone–muscle cross-talk, via osteocalcin and irisin. Importantly, bones contain within their marrow the cradle of the immune system: the anatomical niche where immune stem cells reside, constantly producing high hierarchy immune progenitor cells, which are later released in the blood. Muscles, on the other hand, are essential for metabolism regulation, especially glucose homeostasis, being the largest "organ" with high metabolic activity in the body. Moreover, muscles regulate blood pressure while contracting, and serve important endocrine and immune functions by secreting several bioactive molecules with hormone and cytokine activity. Lastly, the musculoskeletal system as a whole is essential for a variety of physiological processes that require moving parts of the body relative to the whole, such as breathing, which is possible thanks to the coordinated movement of the rib cage and the diaphragm, swallowing, chewing, vision, and many others.

Proteoglycans are abundant in all tissues of the musculoskeletal system, especially those with a high extracellular matrix component such as cartilage, bone, and tendons. Several chondroitin sulfate proteoglycans (CSPGs) and heparan sulfate proteoglycans (HSPGs) are present in the musculoskeletal system where they serve many functions, from structural support to cell signaling, cell adhesion, and inflammation (Alini and Roughley 2001; Brandan and Gutierrez 2013; Fisher et al. 2006; Waggett et al. 1998).

CSPGs and HSPGs contain a relatively small core protein which is decorated with large, linear glycosaminoglycan (GAG) chains composed of repeating disaccharide units of *N*-acetylglucosamine (HS) or *N*-acetylgalactosamine (CS) followed by a glucuronic acid, which in HS can be also converted to its enantiomer iduronic acid. HS and CS vary in their disaccharide composition, chain length, and degree of modification. HS is more variable than CS, both at the level of chain length and for the number and frequency of specific sulfation and acetylation patterns (Rudd et al. 2010). The composition of HS chains in a given tissue at a given developmental stage varies depending on the expression of HS biosynthetic enzymes. There are at least 24 enzymes involved in the biosynthesis of HS in mammals, which can be expressed at various degrees and are subject to various levels of regulation

(Chanalaris et al. 2019). Thus, it is not surprising that HS is the most variable glycosaminoglycan in mammals, which explains their involvement in the fine tuning of an extremely wide range of biological processes (Turnbull et al. 2001). Indeed, increasing evidence suggests that specific structures of HS are required for protein interaction and modulation of signaling pathways (Bernfield et al. 1999; Gao et al. 2016; Ghadiali et al. 2017; Guimond and Turnbull 1999). Heparan sulfate proteoglycans can be secreted and become integral components of the extracellular matrix, such as perlecan (HSPG2), agrin, and collagen XVIII, or can be associated with cells, such as the glypicans, which are tethered to the plasma membrane via a GPI anchor, and the syndecans that are type I transmembrane proteoglycans.

In this chapter, we provide an introduction to the structure and function of syndecans, and then focus specifically on syndecan-3 and its role in development, health, and disease of the musculoskeletal system.

7.2 Syndecans

Members of the syndecan family are type-I transmembrane HSPGs that are ubiquitously expressed, in various assortments, on the cell surface of adherent cells. Syndecans were first discovered in 1983 by Rapraeger and Bernfield but only named a few years later while investigating heparan sulfate- and chondroitin sulfate-containing cell surface proteins (Rapraeger and Bernfield 1983; Saunders et al. 1989). The name syndecan is derived from the Greek term *syndein* meaning "to bind together," as it was thought that these proteins were important for cell adhesion to the extracellular matrix (Rapraeger et al. 1986; Saunders and Bernfield 1988; Saunders et al. 1989). Invertebrates only have one syndecan gene while vertebrates can have up to four: Syndecan-1, -2, -3, and -4 (Fig. 7.1) (Bernfield et al. 1999; Couchman 2003). Since they were first discovered, the syndecans have had alternative names: SDC1 is also known as simply syndecan and CD138, SDC2 is also known as fibroglycan and CD362, SDC3 is also known as *N*-syndecan, SDC4 is also known as ryudocan and amphiglycan.

The expression pattern of each of the mammalian syndecans varies by tissue and stage of development: SDC1 is primarily found in epithelial and mesenchymal tissues; SDC2 in mesenchymal, liver, and neuronal tissues; SDC3 is mostly neuronal but is expressed in some musculoskeletal tissues, macrophages, and blood vessels; and lastly, SDC4 is present in several different tissue types, which makes SDC4's expression unique compared with SDC1, -2, and -3 (Carey et al. 1992; Couchman 2003; David et al. 1992; Marynen et al. 1989; Saunders et al. 1989).

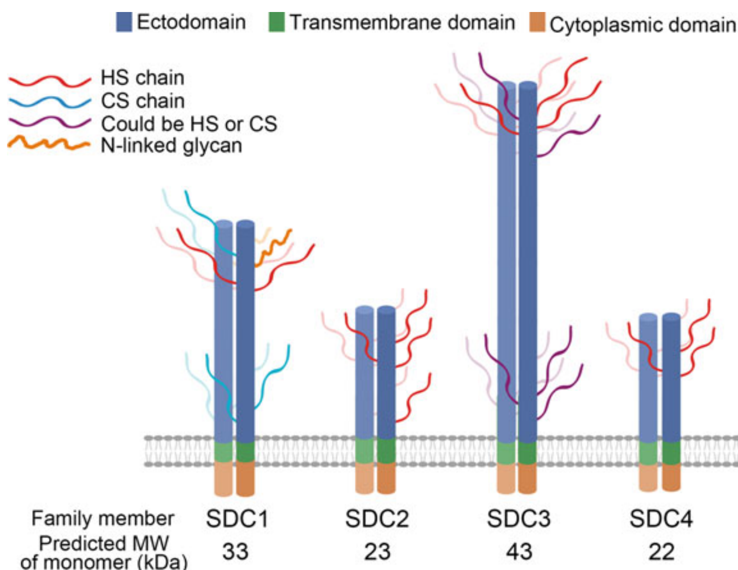

Fig. 7.1 The four human syndecans share structural similarities and can be divided into two subfamilies. SDC1 and SDC3 comprise one syndecan subfamily and are distinct from SDC2 and SDC4 because they have: (1) larger ectodomains, (2) more GAG chains, (3) based on sequence analysis it is postulated that they can harbor both CS and HS chains, and (4) they can harbor additional N- and O-linked glycosylations different from glycosaminoglycan chains. Since it is difficult to experimentally ascertain which sites on SDC1 and SDC3 harbor a CS chain and which sites harbor a HS chain, the likelihood for each site to harbor either type of GAG is evidenced in the figure and based on sequence and similarity analysis. All syndecans can dimerize in vivo, in the figure one of the monomers is shaded to evidence the potential for existing both as monomers and as dimers

7.2.1 Syndecan Structure

Syndecans share a common structure consisting of a larger ectodomain where the GAG chains attach, a transmembrane domain and a short intracellular domain. The cytoplasmic and transmembrane domains are highly conserved whereas the ectodomains vary considerably across the family. Interestingly, the ectodomains and variable regions of syndecan orthologs are highly conserved across vertebrates, suggesting that important specific functions are carried out by these domains. Based on sequence homology SDC1 and SDC3 form a subfamily and SDC2 and SDC4 form another. Additionally, SDC1 and SDC3 can bear both heparan sulfate and chondroitin sulfate chains as opposed to SDC2 and SDC4, which only have heparan sulfate chains (Bernfield et al. 1999). Little structural information is available on the syndecan core proteins, particularly for the ectodomains as these are predicted to be intrinsically disordered (Leonova and Galzitskaya 2015). Only the structure of the SDC4 cytoplasmic domain, in complex with syntenin, is known and

shown to adopt a symmetrical twisted clamp dimer (Choi et al. 2016; Shin et al. 2001).

Although syndecan core proteins are fairly small, ranging between 22 and 43 kDa (Fig. 7.1), they often appear on SDS-PAGE almost two and a half times larger than their predicted molecular weight. This is likely due to their strong SDS-resistant dimerization which is mediated by a region covering the transmembrane domain and a small amino acid sequence within the ectodomain (Asundi and Carey 1995; Couchman 2003). In vitro studies using deletion mutants and recombinant transmembrane domains suggest that a GxxG dimerization motif, contained in the transmembrane domain of all syndecans, has the intrinsic capacity to induce both homo- and heterodimerization, as well as oligomerization (Asundi and Carey 1995; Choi et al. 2010; Dews and Mackenzie 2007). The ectodomain of all syndecans can be proteolytically cleaved at a juxtamembrane site, releasing the ectodomain from the cell surface in a highly regulated process called shedding (Asundi et al. 2003; Choi et al. 2012; Fitzgerald et al. 2000; Hayashida et al. 2008b; Hooper et al. 1997). When the ectodomains are shed they become soluble HSPGs that can act in a paracrine or autocrine manner to either promote or inhibit various signaling pathways. For example, since shedding reduces the number of syndecan proteins on the cell surface, it may inhibit cell signaling through membrane-bound syndecan receptors or it may remove syndecan-mediated inhibition of cell signaling. Moreover, the soluble ectodomain may compete with the cell surface-bound syndecans for ligands (Hayashida et al. 2008a; Steinfeld et al. 1996). Syndecan shedding occurs constitutively in some cultured cells, as part of normal syndecan turnover, but can also be regulated by a plethora of physiological and pathological processes (Manon-Jensen et al. 2010; Yanagishita and Hascall 1984; Zhang et al. 2016, 2017).

The conserved syndecan cytoplasmic domain is less than 40 amino acids long and is divided into three parts: two conserved regions (C1 and C2), which are highly conserved between the syndecans, flanking a variable region (V), which is unique to each syndecan but highly conserved across orthologs (Fig. 7.1). The function of the variable region in the cytoplasmic domain is not well characterized; however, some information is available for SDC1, SDC2, and SDC4 (Chakravarti et al. 2005; Horowitz et al. 1999; Jang et al. 2017; Oh et al. 1997b).

7.2.2 *Syndecan Functions*

Syndecans are involved in the regulation of a vast number of biological processes, possibly due to the high variability of the GAG chains in addition to their core proteins (Turnbull et al. 2001). Syndecans can bind to ECM components, growth factors, cell adhesion molecules, lipases, chemokines, and pathogens. As a result, syndecans act as receptors and co-receptors to modulate biological functions as diverse as cell proliferation, differentiation, adhesion, migration, vesicular trafficking, inflammation, development, and regeneration among many others (Choi et al. 2011; Morgan et al. 2007; Tkachenko et al. 2005).

Both the core syndecan protein and the heparan sulfate chains are important for syndecan-mediated signaling. When the requirement of heparan sulfate in various signaling pathways was discovered it became commonplace to believe that the syndecan core proteins primarily acted as a scaffold for the GAG chains, which were considered the biologically active portion of the proteoglycan. However, more recently this attitude has changed as all the core syndecan proteins have shown biological activity, independent of their GAG chains. All four mammalian core syndecan ectodomains have shown cell adhesion/migration regulatory properties (Beauvais and Rapraeger 2003; De Rossi and Whiteford 2013; Whiteford et al. 2007; Whiteford and Couchman 2006). The addition of a deglycosylated SDC2 ectodomain to cultured colon cancer cells inhibits epidermal growth factor (EGF)-mediated MAPK activation, promotes cell cycle arrest, and inhibits tumor cell adhesion to the ECM (Park et al. 2002). Moreover, cooperation of the core protein and GAG chains also appears to be important. Indeed, cell surface SDC3 has a higher affinity for thrombospondin-1 compared to free heparan sulfate (Herndon et al. 1999). Similar behavior was observed when syndecan/fibronectin interactions were studied (Tumova et al. 2000).

7.3 Syndecan-3

SDC3 is the largest and least characterized among the four syndecans. Based on gene sequence homology, it has been postulated that SDC3 and the other subfamily member, SDC1, have evolved first from the common ancestor syndecan found in invertebrates through gene duplication. Later, the other syndecan subfamily, composed of SDC2 and SDC4, further diverged (Chakravarti and Adams 2006; Spring et al. 1994).

The ectodomain of SDC3 contains eight potential attachment sites for GAGs, either HS or CS, which are identified by a serine–glycine motif surrounded by acidic residues and are clustered in two regions (Figs. 7.1 and 7.2) (Berndt et al. 2001; Carey et al. 1997; Gould et al. 1992). There is no exact consensus motif to confidently predict whether HS or CS will occupy these GAG attachment sites, certain features such as a tryptophan near SG repeating dipeptides may favor HS (Esko and Zhang 1996; Noborn et al. 2016). Additionally, the SDC3 ectodomain is unique among the four syndecans as it contains a large Ser/Thr-rich (mucin-like) domain in the central region, where various kinds of O-linked glycosylation events can occur (Carey et al. 1997; Gould et al. 1992) and Fig. 7.2). At least two soluble matrix metalloproteinases (MMP-2 and MMP-9) and an A-disintegrin and metalloproteinase (ADAM17/TACE) can shed the ectodomain of SDC3, both in vitro and in vivo (Endo et al. 2003; Manon-Jensen et al. 2013; Pruessmeyer et al. 2010). A functional role for SDC3 shedding in both physiological and pathological processes is strongly supported by several findings, including: (1) shed SDC3 ectodomain accumulates in the serum of patients with rheumatoid arthritis (Eustace et al. 2019); (2) shed SDC3 ectodomain accumulates in an

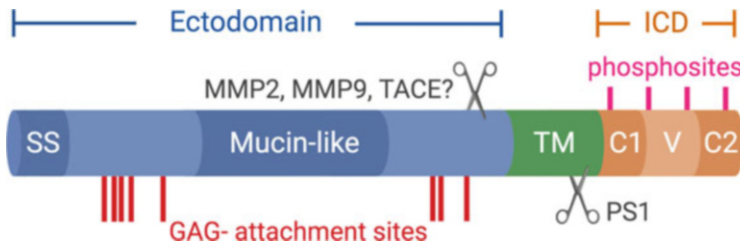

Fig. 7.2 Domain structure and posttranslational modification features of SDC3 core protein. Up to eight GAG-attachment sites have been predicted for at the indicated locations on the ectodomain. The mucin-like domain, in the central region of the ectodomain, can harbor O-linked glycosylations different from glycosaminoglycan chains. The intracellular domain (ICD) contains four potential phospho-sites, all being tyrosine residues

age-dependent manner during nerve development (Asundi et al. 2003); (3) regulation of SDC3 shedding is strongly linked to regulation of appetite and food intake via the melanocortin receptor 4 pathway (Reizes et al. 2003).

The transmembrane domain of SDC3 contains the conserved GxxxG motif mentioned above that mediates dimerization and oligomerization; however, in SDC3, the GxxxG is necessary but not sufficient for dimerization. Instead, another motif in the ectodomain region flanking the transmembrane domain is necessary as well, which includes the last four amino acids of the extracellular domain (ERKE) and the transmembrane domain (Asundi and Carey 1995). SDC3 dimerization and oligomerization likely occur in vivo as demonstrated by the fact that various SDS-resistant species of SDC3 are observed by western blotting in many cell types and tissues showing a molecular weight that is far above the predicted molecular weight (Carey et al. 1997; Jung et al. 2020). Although it has also been hypothesized that the gel shift observed in SDS-PAGE might be due, at least in part, to the high number of proline residues present in the ectodomain of SDC3 and which can cause an extended conformation (Bernfield et al. 1992). Oligomerization of SDC3 in cultured cells (ex vivo) has also been shown using cross-linking experiments (Kirsch et al. 2002). However, to this date, we are not aware of any reports of SDC3 oligomerization or heterodimerization in vivo.

The SDC3 transmembrane domain can also be proteolytically cleaved leading to regulation of SDC3-mediated signaling (Bentahir et al. 2006; Schulz et al. 2003). For example, in mouse embryonic fibroblasts, SDC3 intramembrane cleavage mediated by the presenilin–1(PS1)/γ–secretase complex can occur following a primary ligand-induced shedding of the ectodomain. The shed SDC3 transmembrane domain is then cleaved by PS1 leading to the release of the SDC3 intracellular domain into the cytoplasm and ultimately regulating intracellular localization and function of the calcium/calmodulin-dependent serine protein kinase (CASK) (Schulz et al. 2003). Similarly, SDC3 has been found abundantly present in amyloid plaques in the brains of Tg2576 mice, which carry a mutation in the gene encoding the amyloid precursor protein (APP) that increases amyloid-β production and a phenotype resembling that

of patients affected by familial Alzheimer's disease (O'Callaghan et al. 2008). However, in Tg2576 mice SDC3 appears to be associated mostly with plaques rich in amyloid β-peptide 40 (Aβ40) while nearly absent from plaques rich in amyloid β-peptide 42 (Aβ42), which is more fibrillogenic than Aβ40 and therefore considered by many the most toxic species of Aβ in Alzheimer's disease. The finding that, somehow, SDC3 selectively associates with the less toxic species of Aβ, together with the knowledge that both SDC3 and the amyloid precursor protein (APP) are cleaved intramembrane by PS1 after shedding of the ectodomain, is fascinating. Moreover, it has been reported that heparan sulfate regulates β-secretase-mediated cleavage of APP (Scholefield et al. 2003), which poses further questions on a potential role for SDC3 in Alzheimer's disease.

The intracellular domain of SDC3 recapitulates the typical syndecan intracellular domain primary structure, with two variable regions flanking a central constant region (Couchman 2010; Reiland et al. 1996). The intracellular domains of all syndecans have been shown to interact with the cytoskeleton. In the case of SDC3, the C1 region binds to cortactin and tubulin in neuroblastoma cells (Granes et al. 2003; Kinnunen et al. 1998). Heparin-binding growth-associated molecule (HB-GAM, a.k.a. pleiotrophin or osteoblast stimulation factor 1, OSF1) is a SDC3 ligand that induces neurite outgrowth. When HB-GAM is ligated to SDC3 in neuroblastoma cells, SRC kinase and cortactin phosphorylation increase, leading to neurite outgrowth (Granes et al. 2003; Kinnunen et al. 1998).

Although the evidence that syndecans regulate many signaling pathways is overwhelming, it is not clear to this date whether they can also signal autonomously. HB-GAM, artemin, neurturin, and glial cell line-derived neurotrophic factor (GDNF) are some of such ligands that have been postulated to signal autonomously through SDC3 (Bespalov et al. 2011). Receptor phosphorylation in response to ligand binding is a highly conserved and widespread molecular mechanism of signal transduction. Although syndecans have no kinase activity of their own, phosphorylation of their intracellular domains by other kinases has been reported (Asundi and Carey 1997; Horowitz and Simons 1998; Oh et al. 1997a; Ott and Rapraeger 1998). In vitro, SDC3 can be phosphorylated by the tyrosine kinase ELK1 on 4 equally spaced tyrosine residues (Asundi and Carey 1997) and by PKC on two serine residues (Prasthofer et al. 1995). However, SDC3 phosphorylation in vivo has not been reported. Strikingly, germline deletion of Sdc3 in mice leads to an overall increase in tyrosine phosphorylation in cultured myoblasts (Cornelison et al. 2004), suggesting a role for SDC3 in controlling the homeostasis of the phosphoproteome at a global level (Jones et al. 2019).

7.4 Syndecan-3 in the Musculoskeletal System

Most of the existing work on the role of SDC3 in the musculoskeletal system has investigated muscle and cartilage, while little attention so far has been dedicated to SDC3's role in bone development and virtually nothing in tendons and ligaments.

Here we will discuss current literature on SDC3 in muscle and cartilage, with a brief reference to bone.

7.4.1 SDC3 in Muscle Development

Vertebrate skeletal muscle develops early in the embryo, mostly arising from the paraxial mesoderm, which then segments into the somites (Christ and Ordahl 1995). As the somites mature the dermomyotome is formed and is the source of muscle progenitor cells expressing the transcription factors Paired box 3 (PAX3) and Paired box 7 (PAX7), which drive muscle progenitor specification (Buckingham 2007; Relaix et al. 2005). PAX3-expressing muscle progenitors are essential for delamination and migration of muscle progenitors from the dermomyotome to the limb buds where they begin to rapidly proliferate becoming myoblasts (Goulding et al. 1994). Terminally differentiated myoblasts then fuse to one another generating multinucleated muscle cells known as primary muscle fibers (Relaix and Zammit 2012; Zammit et al. 2004). This first wave of myogenesis (the process of muscle generation), known as embryonic myogenesis, is then followed by a second wave called fetal myogenesis, which leads to generation of secondary muscle fibers and muscle stem cells. The latter, also known as satellite cells, will then be responsible for a third, and final, wave of myogenesis that covers postnatal muscle development and growth (Biressi et al. 2007).

SDC3 is an important regulator of myogenesis and is expressed dynamically during development (Pisconti et al. 2012). During mouse muscle development, SDC3 is expressed from approximately embryonic (E) day 10.5, both in somites and in the limb bud (Olguin and Brandan 2001). By immunofluorescence analysis, SDC3 colocalizes with myogenin-expressing cells, both in the somite and in the limb bud, but reaches its peak of expression in the limb bud at E13.5, while myogenin expression is still increasing, suggesting an important role for SDC3 in the early phases of myogenic differentiation (Olguin and Brandan 2001). Later during mouse development, SDC3 levels in limb muscles progressively decrease remaining restricted to satellite cells and, possibly, endothelial cells during postnatal life (Cornelison et al. 2001).

7.4.2 SDC3 in Muscle Regeneration, Aging, and Disease

Muscle stem cells (MuSCs), also known as muscle satellite cells, were discovered in 1961 in the context of electron microscopy analysis of frog muscle, and immediately hypothesized to be the source of regenerating fibers in injured muscle (Mauro 1961). The first observations of MuSCs also suggested that they might exist in a mitotically quiescent state, due to extremely low abundance of cytoplasm and condensed chromatin structure. Indeed, we know today that after muscle growth has ended,

usually at the end of puberty, MuSCs become quiescent in the vast majority of muscles, where they will only rarely reactivate and reenter the cell cycle (Pawlikowski et al. 2015). Upon activation, MuSCs rapidly undergo a number of metabolic, transcriptional, and translational changes that lead to cell cycle reentry and to increased levels of key transcription factors called muscle regulatory factors (MRFs). The MRFs, namely MyoD1, Myf5, myogenin, and MRF4, act in a coordinated manner to drive the transcriptional program leading to muscle differentiation and ultimately function (Mashinchian et al. 2018; Olguín and Pisconti 2012; Schmidt et al. 2019).

In postnatal mouse muscle, SDC3 protein and mRNA are detectable in quiescent MuSCs. SDC3 mRNA is then strongly and rapidly induced upon MuSC activation (Cornelison et al. 2001; Tanaka et al. 2009; van Velthoven et al. 2017) and peaks around the onset of differentiation (Casar et al. 2004; Cornelison et al. 2004), suggesting a role for SDC3 in the quiescent and proliferating states of MuSC life cycle. Consistent with this hypothesis Cornelison et al. found in 2004 that the number of MuSCs per isolated muscle fiber was greatly increased compared to wild type controls, and that this was associated with an increase in the percentage of centrally nucleated muscle fibers in muscle sections of Sdc3 null (*Sdc3*$^{-/-}$) mice (Cornelison et al. 2004).

Muscle fiber nuclei, also called myonuclei, are normally positioned at the periphery of muscle fibers, squashed underneath the plasma membrane by the compact packaging of contractile structures known as sarcomeres. Conversely, myonuclei are positioned in the center of newly formed and regenerating muscle fibers, which is why the presence of centrally nucleated muscle fibers is considered a hallmark of muscle regeneration as its frequency is extremely low in uninjured, healthy muscle. In certain medical conditions, such as muscular dystrophy, where muscle tissue undergoes continuous cycles of injury and regeneration, a large fraction of muscle fibers are centrally nucleated (Mashinchian et al. 2018). Thus, the presence of a significant number of centrally nucleated muscle fibers in uninjured *Sdc3*$^{-/-}$ mice was originally interpreted by Cornelison et al. as a novel form of muscular dystrophy (Cornelison et al. 2004). It was then startling for us to observe, approximately a decade later, that crossing *Sdc3*$^{-/-}$ mice to dystrophic mice sharing the same C57Bl/6 genetic background (*mdx4cv* mice) did not lead to a worsening of the dystrophic phenotype in the double mutants, but rather to an improvement (Pisconti et al. 2016). In reality, we now know that Sdc3 loss in mice does not cause a novel form of muscular dystrophy as previously thought, it leads to altered MuSC dynamics which in turn leads to enhanced muscle regeneration (Pisconti et al. 2016; Pisconti et al. 2010).

The apparently conflicting interpretations of the same phenotype are easily reconciled when the dynamics of MuSC progression through their life cycle are all taken into account (Fig. 7.3). SDC3 appears to play a role in maintaining MuSC quiescent in uninjured muscle, through a PAX7-dependent mechanism that is not completely understood yet (Pisconti et al. 2010, 2016). Indeed, the finding that freshly isolated muscle fibers harbor more activated MuSCs, as measured by both MyoD1 expression (Pisconti et al. 2010) and SDC4 expression (Cornelison et al.

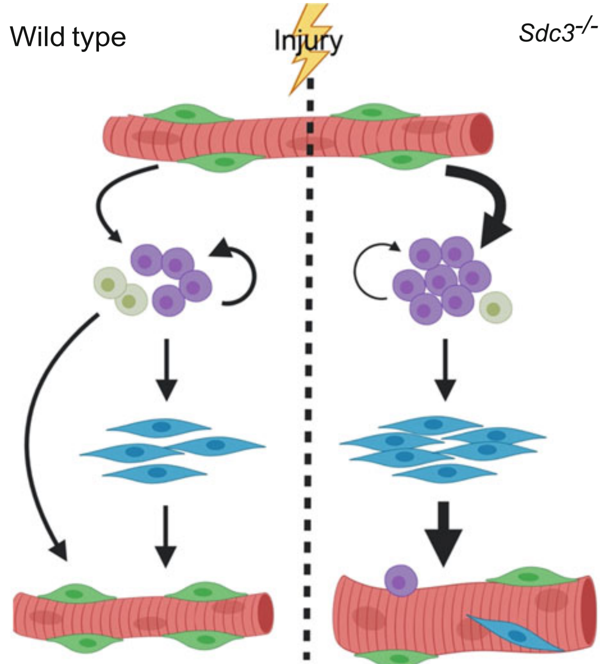

Fig. 7.3 Schematic of the muscle regeneration phenotypes observed in *Sdc3*$^{-/-}$ mice. SDC3 promotes MuSC quiescence and proliferation. In its absence, MuSCs are more prone to activation and fail to return quiescent upon activation. Since proliferation is slower in the absence of SDC3 and fusion is enhanced, a pool of slowly proliferating and readily fusing myoblasts is maintained throughout life and contributes to myofiber hypertrophy and hyperplasia in regenerated muscles and improved muscle health in aging and muscular dystrophy

2004) is consistently observed in both reports. In the absence of SDC3, MuSCs become more readily activated in response to a stimulus and/or fail to return to quiescence after activation (Pisconti et al. 2010). This leads to a greater number of MuSCs becoming activated and fusing to uninjured muscle fibers during normal homeostasis maintenance and, consequently to a greater number of centrally nucleated fibers. Additionally, we have shown that the impairment in MuSC return to quiescence due to SDC3 loss maintains a pool of activated, slowly dividing myoblasts (Pisconti et al. 2016 and Fig. 7.3). This greater pool of myoblasts eventually leads to the generation of larger muscle fibers over time in acutely injured muscle and in repeatedly injured muscle, as well as to an enhanced regenerative response in dystrophic muscle (Pisconti et al. 2010, 2016).

Another important role for SDC3 in MuSC is the regulation of cell cycle progression, likely via multiple signaling pathways. We have shown that SDC3 promotes cleavage and activation of the Notch1 receptor in MuSCs. Although it is not known whether SDC3 and Notch1 directly interact, they are present in the same complex on the plasma membrane of myoblasts where SDC3 promotes Notch1 signaling activation by facilitating its TACE-mediated extracellular cleavage (Fig. 7.4). Additionally, it is possible that SDC3 and Notch1 are simultaneously cleaved by the gamma-secretase complex. Notch1 signaling which in turn promotes both MuSC timely progression through the cell cycle and self-renewal (Pisconti et al. 2010). In the absence of SDC3, Notch1 signaling in MuSCs is impaired, therefore self-renewal is impaired, cell cycle progression is slower and fusion is accelerated

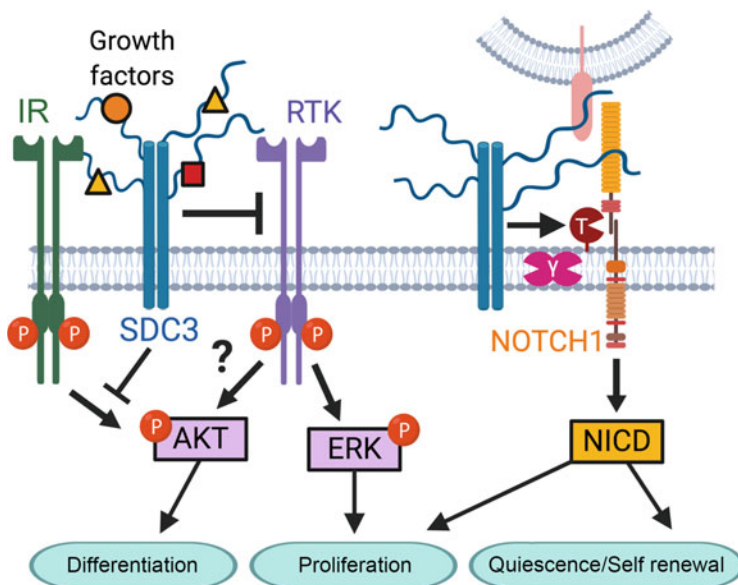

Fig. 7.4 Schematic of known signaling pathways regulated by SDC3 in MuSCs and myoblasts. SDC3 promotes Notch1 signaling by facilitating its extracellular cleavage mediated by ADAM17/TACE (T). Additionally, SDC3 blocks RTK signaling a consequent growth factor-induced activation of ERK1/2, presumably by sequestering growth factors through its GAG chains, although the latter mechanism is not verified. Whether RTK-induced activation of AKT is also inhibited by SDC3 is not known

(Figs. 7.3 and 7.4). Reduced Notch1 signaling is also accompanied by increased growth factor signaling through ERK1/2 (Cornelison et al. 2004), which prevents MuSC depletion due to Notch signaling impairment and in fact contributes to the maintenance of large pool of myoblasts with enhanced fusion properties. This pool of readily fusing myoblasts is then likely to be responsible for all the phenotypes observed in vivo, which include: (1) presence of centrally nucleated myofibers in uninjured muscle, due to MuSCs that become activated throughout life and then fail to return quiescent (Cornelison et al. 2004); (2) myofiber hyperplasia in uninjured *Sdc3*$^{-/-}$ muscle (Cornelison et al. 2004); (3) generation of larger myofibers containing more myonuclei in response to single, repeated and chronic injury (Pisconti et al. 2010, 2016); (4) presence of myogenin+ cells in uninjured muscle of *Sdc3*$^{-/-}$ mice (Pisconti et al. 2016); and (5) ameliorated muscle health in old *Sdc3*$^{-/-}$ mice (Pisconti et al. 2016).

More recently, a global phosphoproteomics study revealed a role for SDC3 in the balance between proliferation and differentiation of myoblasts in vitro. Heavy depletion of SDC3 in a myoblast cell line caused aberrant signaling in several tyrosine kinase-related pathways, most notably the insulin signaling pathway. Furthermore, the insulin receptor immunoprecipitated with SDC3, suggesting these

molecules are in a complex on the cell membrane. Mechanistic insights propose that SDC3 inhibits INSR/AKT signaling and promotes proliferation MuSCs during the early stages of myogenesis, but as MuSCs begin to differentiate and downregulate SDC3, this blockade is lost and INSR/AKT signaling increases enhancing myoblast differentiation and fusion (Jones et al. 2021).

During aging and even in the absence of any significant disease, skeletal muscle undergoes substantial loss of mass and strength, a process known as sarcopenia. In mice, MuSC contribution to uninjured muscle fibers declines over time but remains significant, especially in muscles of the upper body such as the diaphragm or the extraocular muscle (Pawlikowski et al. 2015). However, the contribution of MuSCs to sarcopenia is debated and possibly not highly significant as evidenced by MuSC depletion experiments (Fry et al. 2015; Keefe et al. 2015). Aged *Sdc3*$^{-/-}$ mice show reduced muscle fibrosis compared to aged wild-type mice that are associated with an increase in differentiated myoblasts and centrally nucleated muscle fibers (Pisconti et al. 2016). Although no information is available on the degree of sarcopenia in aged *Sdc3*$^{-/-}$ mice, it is possible that the increased number of myoblasts is directly responsible for the observed decrease in fibrosis, since MuSCs and myoblasts have been shown to secrete exosomes that directly repress fibroblast expansion and consequently ECM deposition and fibrosis (Fry et al. 2017). Crucially, SDC1, which shares significant homology with SDC3, has been reported to play important roles in vesicle trafficking and exosome generation (Baietti et al. 2012). It is also important to consider that the *Sdc3*$^{-/-}$ mice used in all the work cited so far are germline knockout mice and is therefore possible that the muscle phenotypes observed are not entirely due to MuSCs. SDC3 is also expressed in endothelial cells where the SDC3 ectodomain inhibits angiogenesis (Chen et al. 2004; De Rossi and Whiteford 2013). Indeed *Sdc3*$^{-/-}$ muscles show an increase in capillary density compared to wild-type muscles, which might also contribute to the improvement in muscle health observed in aged *Sdc3*$^{-/-}$ mice (Pisconti et al. 2016).

7.4.3 SDC3 in Cartilage Development, Regeneration, and Disease

Cartilage is a unique hypocellular, aneural, alymphatic, avascular loadbearing tissue, composed of only one cell type, chondrocytes, which are sparsely embedded within an extracellular matrix (ECM) of collagens, proteoglycans, and non-collagenous proteins (Buckwalter and Mankin 1998). Cartilage functions to enable the smooth frictionless articulation of joint surfaces and to resist the mechanical loading in daily activities. The various elements of the cartilage ECM are interconnected and differ in their relative amounts and distribution between the areas around chondrocytes, known as territorial matrix, and the areas between territorial areas, known as interterritorial matrix (Pap and Bertrand 2013).

Chondrocytes are specialized, metabolically active cells responsible for the formation of the macromolecular framework of the cartilage ECM. This occurs by producing collagen, proteoglycans, and non-collagenous proteins (Buckwalter and Mankin 1998). Chondrocytes maintain the integrity of the cartilage ECM and are able to adapt to various physical stimuli, such as loading and stress, by altering their functional demands (Handley and Lowther 1979). In adult articular cartilage chondrocytes are quiescent. In osteoarthritis or when the cartilage tissue is mechanically injured, chondrocyte proliferation can be reactivated (Buckwalter and Mankin 1998).

Proteoglycans are not only expressed between chondrocytes in the ECM, but also on the surface of chondrocytes (Pfander et al. 2001). All four syndecans are expressed in chondrocytes. SDC3 is considered a marker of early chondrocyte differentiation (Molteni et al. 1999), however, its mechanistic roles during cartilage development are unknown. In addition to being expressed in chondrocytes during development, SDC3 has also been detected in the synovium of adult human joints, suggesting a role for SDC3 in maintaining cartilage homeostasis in postnatal life (Patterson et al. 2005). In addition, SDC3 is involved in limb morphogenesis, skeletal development, and later regeneration (Kosher 1998). Recently we have demonstrated a dual role for SDC3 being pro-inflammatory in the joint and anti-inflammatory in the skin, contributing to the idea that SDC3's role may be tissue or inflammatory state specific (Kehoe et al. 2014).

Osteoarthritis (OA) is the most common joint disease in the world. OA causes severe pain and stiffness of the joints, which make the patient less able to go about their daily physical activities. OA is characterized by progressive deterioration and loss of articular cartilage, inflammation of the synovium, formation of osteophytes, and an increase in subchondral bone mass. In healthy articular cartilage, chondrocytes are responsible for maintaining a balance of anabolic and catabolic pathways to preserve the health and function of the joint. This balance is disturbed in OA. Signs of cartilage damage in OA include fibrillations of the superficial layer, a progressive loss of proteoglycans and appearance of mitotic cell divisions and chondrocyte death (Loeser et al. 2012). During OA progression, chondrocytes undergo hypertrophic differentiation and actively participate in disease development by losing their ability to maintain the cartilage ECM. Specifically, chondrocytes in OA contribute to matrix degradation, abnormal matrix production, undergo increased proliferation, and increased apoptosis (Sherwood 2019).

The percentage of chondrocytes expressing SDC3 significantly increases (from 20% to more than 80%) in the upper zone of OA articular cartilage compared to healthy articular cartilage (Pfander et al. 2001). Annexin VI is a marker of late chondrocyte differentiation and annexin VI expressing cells are involved in Ca2+ homeostasis. Similar to SDC3+ chondrocytes, also the number of annexin VI expressing cells is significantly increased in the upper zones of OA cartilage, as well as the number of proliferating cells, identified as PCNA positive (Pfander et al. 2001). Thus, it is possible that the increased expression of SDC3 in OA chondrocytes is part of a proliferative response to OA-induced chondrocyte death. A recent study has further confirmed the increase in SDC3 expression in OA

cartilage (Chanalaris et al. 2019), however, the nature of the functional link between SDC3 and chondrocyte maturation and growth in articular cartilage has not been established yet.

Inflammation is a central feature of rheumatoid arthritis (RA) that affects around 1% of the population and can result in disability and morbidity (McInnes and Schett 2011). In RA, inflammation of the synovial membrane is characterized by infiltration and activation of leukocytes, which in turn can result in progressive destruction of cartilage and bone. Several chemokines are involved in facilitating this inflammatory response and their expression levels are elevated in serum, synovial fluid, and synovial membrane of patients affected by RA (Szekanecz and Koch 2016). HSPG expression changes in human RA synovium have been reported, which include an increase in SDC3 expression (Patterson et al. 2008). This increase in SDC3 expression is accompanied by the selective induction of a CXCL8 chemokine-binding site associated with SDC3 in endothelial cells (Patterson et al. 2005), suggesting a key role for SDC3 in the regulation of the inflammatory response in RA. Indeed, using a mouse model of RA, we have shown that SDC3 increases leukocyte accumulation and disease severity (Kehoe et al. 2014). Thus, we have proposed that membrane-bound endothelial SDC3 presents chemokines to blood leukocytes resulting in leukocyte trafficking into RA synovium (Kehoe et al. 2014). Moreover, an article recently published by Eustace et al. characterizes the role of soluble SDC3 in inflammation using in vitro and in vivo models (Eustace et al. 2019). The authors show that soluble SDC3 binds chemokines, reduces leukocyte migration in vitro and ameliorates disease severity in antigen- and collagen-induced in vivo arthritis mouse models. The observed anti-inflammatory mechanisms of shed SDC3 appear to be associated with competitive binding of SDC3 to chemokines, which limit their availability to recruit inflammatory cells. This study demonstrates that the addition of soluble SDC3 may represent an alternative therapeutic approach for the treatment of inflammatory diseases such as RA. Consistently, we have shown in a recent work that transplantation of *Sdc3*$^{-/-}$ mesenchymal stem cells (MSCs) in the knee of an antigen-induced mouse model of RA, accelerates healing likely by decreasing inflammation. This anti-inflammatory effect of *Sdc3*$^{-/-}$ MSCs in vivo is associated with reduced adhesion in vitro to a collagen substrate, which is not observed with other substrates, indicating a direct role for SDC3 as a collagen receptor in MSCs. Moreover, this reduced adhesion to collagen was associated with increased AKT signaling and decreased ERK1/2 signaling in *Sdc3*$^{-/-}$ MSCs, altogether suggesting that transplanted MSCs use SDC3 to navigate the synovium and respond to adhesion signaling by activating the ERK1/2 pathway, which then somehow mediates to SDC3-led regulation of inflammation (Jones et al. 2020).

7.4.4 SDC3 in Osteogenesis

Endochondral ossification is a complex, tightly controlled embryonic development process, which is responsible for the formation of the axial skeleton, including the

long bones (Ortega et al. 2004). During endochondral ossification, chondrocytes in the growth plate undergo a series of events, including proliferation, hypertrophy, and terminal differentiation (Mackie et al. 2008).

Studies led by the Pacifici group examined the biology of SDC3 in bone development, mostly using the chick embryo as a model and focusing especially on its role in the growth plate (Kirsch et al. 2002; Koyama et al. 1996; Shimo et al. 2004). The results demonstrated that SDC3 is present on the chondrocyte surface as monomer, dimer, and also in higher hierarchical oligomeric structures (Kirsch et al. 2002; Pacifici et al. 2005). In vivo, SDC3 is strongly expressed in the proliferative zone of avian and mammalian growth plates, indicating that SDC3 functions in limb skeletal development is conserved (Pacifici et al. 2005). Interestingly, a study in Chinese beef cattle breeds found that polymorphisms in SDC3 are associated with height, length, and cannon bone circumference, suggesting that SDC3 in the growth plate may control growth (Huang et al. 2016). SDC3 expression in developing bone increases dramatically as early as during chondrification of the mesenchymal condensation and later decreases along the diaphysis while remaining high in the epiphyseal articular cap and along the metaphysis (Koyama et al. 1996). SDC3 selectively regulates chondrocyte proliferation and maturation in the growth plate in an Indian hedgehog (Ihh)-dependent manner (Shimo et al. 2004). Moreover, both in vivo and in vitro studies have shown that SDC3 mediates the specific response of chondrocytes to growth factors such as fibroblast growth factor (FGF-2), without affecting the mitotic activity of heparin-independent growth factors (Kirsch et al. 2002; Shimazu et al. 1996). Importantly, these data have been further corroborated by the independent finding that the Runt-related transcription factor 2 (Runx2), an essential driver of osteogenesis (Schroeder et al. 2005), induces expression of FGF receptors, several proteoglycans, including SDC3, and various heparan sulfate biosynthesis enzymes in immortalized osteogenic progenitors in vitro (Imai et al. 1998; Teplyuk et al. 2009). Another important regulator of osteogenesis is the SDC3 ligand HB-GAM, which also promotes neuronal migration and neurite outgrowth during brain development (Hienola et al. 2006). HB-GAM is expressed around osteocytes in bone and at the growth plate of mice; in an in vivo model of bone injury, both SDC3 and HB-GAM are rapidly and dramatically upregulated in the periosteum at the site of injury, suggesting a role in osteoblast recruitment to the periosteum during the regenerative response to injury (Imai et al. 1998). Three studies have shown that in vivo overexpression of HB-GAM leads to increased bone mass through stimulation of bone formation (Imai et al. 1998; Masuda et al. 1997; Tare et al. 2002b). Furthermore, in in vitro studies, HB-GAM was shown to stimulate osteoblast differentiation, although it inhibited the osteo-inductive effects of BMP-2 (Tare et al. 2002a). Studies on the effects of deleting HB-GAM have given conflicting results. A study by Lehman et al. showed no effect on adult bone volume or bone turnover (Lehmann et al. 2004), while a study by Imai et al. (2009) found a profound effect of HB-GAM deletion on bone, including low bone volume due to low bone formation, and a lack of response to changes in mechanical loading of the bones. Interestingly, both papers concluded that HB-GAM was not required during early development of the skeleton, despite it

being expressed during early intramembranous and endochondral bone formation (Mitsiadis et al. 1995). As SDC3 is known to play an important role in skeletal development, this further supports previous studies showing that SDC3 interacts with factors other than HB-GAM, such as FGF2, Ihh, and BMP-2, to regulate bone development (Pacifici et al. 2005).

We have recently studied the bone phenotype of *Sdc3*$^{-/-}$ mice (Johnson de Sousa Brito et al. 2021). These mice have increased bone volume of the long bones at birth, indicating that SDC3 inhibits bone formation during embryonic development, possibly through inhibition of mesenchymal cell aggregates during the early stages of endochondral bone formation (Seghatoleslami and Kosher 1996). However, postnatally the *Sdc3*$^{-/-}$ mice show a reduced longitudinal growth rate and develop a low bone volume phenotype. At 3 months of age, trabecular bone volume and cortical thickness are significantly reduced, leading to decreased bone strength. The phenotype is mostly due to a defect in bone formation. This appears to be a cell-autonomous defect in osteoblasts, as in vitro, *Sdc3*$^{-/-}$ osteoblast differentiation and activity are decreased. The underlying mechanism is at least partially due to decreased protein levels of the WNT receptor Frizzled1 (FZD1), leading to a decrease in WNT signalling. As mRNA levels of Fzd1 are not affected in *Sdc3*$^{-/-}$ osteoblasts, the data suggest that SDC3 is required for protein stability of FZD1.

Although very little is known about the role of SDC3 in osteoclast biology, a recent study found an effect of SDC3, as well as the other three syndecans, on osteoclast differentiation. The addition of recombinant syndecan ectodomains appears to inhibit osteoclast formation and bone resorption in vitro except for the SDC3 ectodomain, which has no effect on bone resorption in contrast to the ectodomains of SDC1, 2, and 4 (Kim et al. 2018). It is currently unknown if this effect is important in vivo, however, all syndecans are known to undergo shedding, and this could release these exodomains into the bone environment. In contrast, deletion of the SDC3-ligand HB-GAM resulted in reduced osteoclast numbers in transgenic mice (Imai et al. 2009). Furthermore, our recent studies on the *Sdc3*$^{-/-}$ mouse showed decreased osteoclast formation, both in vivo and in vitro, although osteoclast activity was increased in vitro in *Sdc3*$^{-/-}$ osteoclasts (Johnson de Sousa Brito et al. 2021). This may indicate that SDC3-dependent mechanisms may both stimulate and inhibit osteoclast differentiation and function, respectively.

7.4.5 Concluding Remarks

The musculoskeletal system is a complex anatomic structure that serves the purpose of supporting life at many levels. SDC3 is a similarly complex biomolecule, orchestrating transduction and regulation of many signaling pathways in the various elements that compose the musculoskeletal system. The biochemistry and biology of SDC3 have been so far overlooked, leaving a gap in our understanding of development, health, and disease of the musculoskeletal system. *Sdc3*$^{-/-}$ mice show a whole range of interesting phenotypes, not only in the musculoskeletal system but also in

other contexts, such as development of the nervous system and regulation of metabolism. However, our understanding of the molecular mechanisms that involve SDC3 has been hampered so far by the lack of tissue- and time-specific knockout or other types of transgenic mice. With the extraordinary advancement in molecular genetics brought about by CRSPR/Cas technology, powerful new tools will soon become available and will enable tackling of questions about SDC3's biology that are still open today. We look forward to a new wave of interest in SDC3 to unfold. Stay tuned.

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Chapter 8
Proteoglycans of the Neural Stem Cell Niche

Lars Roll and Andreas Faissner

Abstract Neural stem cells (NSCs) of the central nervous system (CNS) follow a precisely timed sequence of neurogenesis and gliogenesis, before they generally vanish after birth. In the adult CNS residual NSCs can be detected in two neurogenic regions, the subependymal zone (SEZ) of the lateral ventricle and the subgranular zone (SGZ) in the dentate gyrus of the hippocampus. These regions present favorable environments for stem cells and are considered privileged niches for their persistence and differentiation. Niches comprise the stem cells, their progeny, specialized niche cells, and a specialized extracellular matrix (ECM) environment. Morphogens, cytokines, hormones, and glycoproteins contribute to a rich microenvironment that guides self-renewal and/or differentiation of stem cells. It has become evident that a subclass of ECM constituents is of particular importance for the structure and function of the stem cell niche, the proteoglycans (PGs). PGs can embody a considerable structural variability and intervene in many important cellular functions such as cell proliferation, migration, and differentiation. A better understanding of niche PGs and their regulatory roles will be valuable for the design of artificial growth support of stem cells. This chapter discusses recent insights into the composition and functions of PGs in the microenvironment of NSCs.

List of Abbreviations

ChABC	Chondroitinase ABC
CNS	Central nervous system
CS	Chondroitin sulfate
CSC	Cancer stem cell
CSPG	Chondroitin sulfate proteoglycan
DS	Dermatan sulfate

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GAG	Glycosaminoglycan
GBM	Glioblastoma multiforme
GPC	Glypican
HS	Heparan sulfate
HSPG	Heparan sulfate proteoglycan
KS	Keratan sulfate
Mab	Monoclonal antibody
NSC	Neural stem cell
OPC	Oligodendrocyte precursor cell
oSVZ	Outer subventricular zone
PG	Proteoglycan
PNN	Perineuronal net
RG	Radial glia
RPTP	Receptor protein tyrosine phosphatase
SDC	Syndecan
SEZ	Subependymal zone
SVZ	Subventricular zone
TMZ	Temozolomide

8.1 Stem Cells in the Developing and Adult CNS

The central nervous system (CNS) develops during the embryonic and early postnatal life of the mouse. Starting point is the neuroepithelium that consists of a sheet of symmetrically dividing cells. The neuroepithelium gives rise to the radial glia (RG), the principal stem cell of the developing CNS (Malatesta and Götz 2013). In the course of neurogenesis, the division pattern of radial RG stem cells shifts from a symmetric to an asymmetric mode (Taverna et al. 2014). In successive waves, neuronal and later glial progenitors are generated that build up the growing cortex (Doetsch 2003; Götz and Huttner 2005). The outer or basal radial glia constitutes a further population of neural stem cells that are considered responsible for the gyrification of the cortex during the evolution of primates and humans (Florio and Huttner 2014). In the adult CNS, subpopulations of specialized astrocytes in the subependymal zone (SEZ) of the lateral ventricle and the subgranular zone (SGZ) of the dentate gyrus of the hippocampus serve as slowly dividing stem cells (Kriegstein and Alvarez-Buylla 2009; Ihrie and Alvarez-Buylla 2011). Cells from the SEZ migrate and replace neurons in the olfactory bulb, whereas cells from the SGZ remain in the hippocampus, a brain region that is strongly associated with memory formation. It is assumed that tumors of the CNS such as astrocytomas, gliomas, and glioblastomas comprise cancer stem cells that may derive from neural stem cells (Wang et al. 2010). Neural stem/progenitor cells (NSCs) reside in stem cell niches, specialized environments that consist of NSCs, NSC-derived progenitor cells, niche support cells, blood vessels, and a distinct extracellular matrix (ECM)

microenvironment (Scadden 2006; Rojas-Ríos and Gonzalez-Reyes 2014). The past years have shown that complex ECM milieus partake in the regulation of CNS development and plasticity in response to lesions (Roll and Faissner 2014).

8.2 The Neural Extracellular Matrix

The extracellular matrix (ECM) constructs a complex interactome of glycoproteins, proteoglycans, and complex glycans that are recognized by specific complementary cellular receptors. Systematic bioinformatics analysis has led to the conclusion that the ECM comprises 300 genes designated as matrisome core that includes 200 glycoproteins, 40–45 collagens, and about 35 proteoglycans. The core constituents interact with up to 800 matrisome-associated components (Hynes and Naba 2012; Naba et al. 2012). Proteoglycans (PGs) consist of a protein core and at least one covalently bound glycosaminoglycan (GAG) side chain (Iozzo and Schaefer 2015). The latter defines the PG subclass in dependence on the structure of the GAG. According to this criterion chondroitin sulfate proteoglycans (CSPGs), dermatan sulfate proteoglycans (DSPGs), heparan sulfate proteoglycans (HSPGs), and keratan sulfate proteoglycans (KSPGs) can be distinguished (Fig. 8.1) (Bandtlow and Zimmermann 2000; Barros et al. 2011). As indicated in the scheme, many molecules carry more than one type of GAG, for example, chondroitin sulfate and keratan sulfate GAGs, at the same time. CSPGs are mostly soluble and located in the interstitial space, while HSPGs are often membrane-associated (Faissner and Reinhard 2015; Maeda 2015). While originally studied mainly in connective tissues, increasing evidence supports important biological roles of the ECM in the CNS (Barros et al. 2011; Dityatev et al. 2010b; Garwood et al. 2001; Zimmermann and Dours-Zimmermann 2008; Long and Huttner 2019). Thus, the ECM is involved in the biology of NSCs (Faissner and Reinhard 2015; Theocharidis et al. 2014), the development of the spinal cord (Wiese and Faissner 2015), the development and pathology of the visual system (Reinhard et al. 2015), and in formation and plasticity of synapses (Faissner et al. 2010; Heikkinen et al. 2014; Dzyubenko et al. 2016; Dityatev et al. 2010a; Sorg et al. 2016). The neural ECM has also been recognized as an important obstacle to regeneration in the mammalian CNS (Kwok et al. 2014; Soleman et al. 2013; Silver and Silver 2014).

8.3 The Stem Cell Niche

Stem cells are not ubiquitously distributed but rather reside in highly specialized microenvironments termed niches that are indispensable for their maintenance. These niches comprise the stem cell, its progeny, specialized niche support cells, the vicinity of blood vessels, and also serotonergic input (Rojas-Ríos and Gonzalez-Reyes 2014; Shen et al. 2008; Tong et al. 2014). Thereby, niches can integrate local

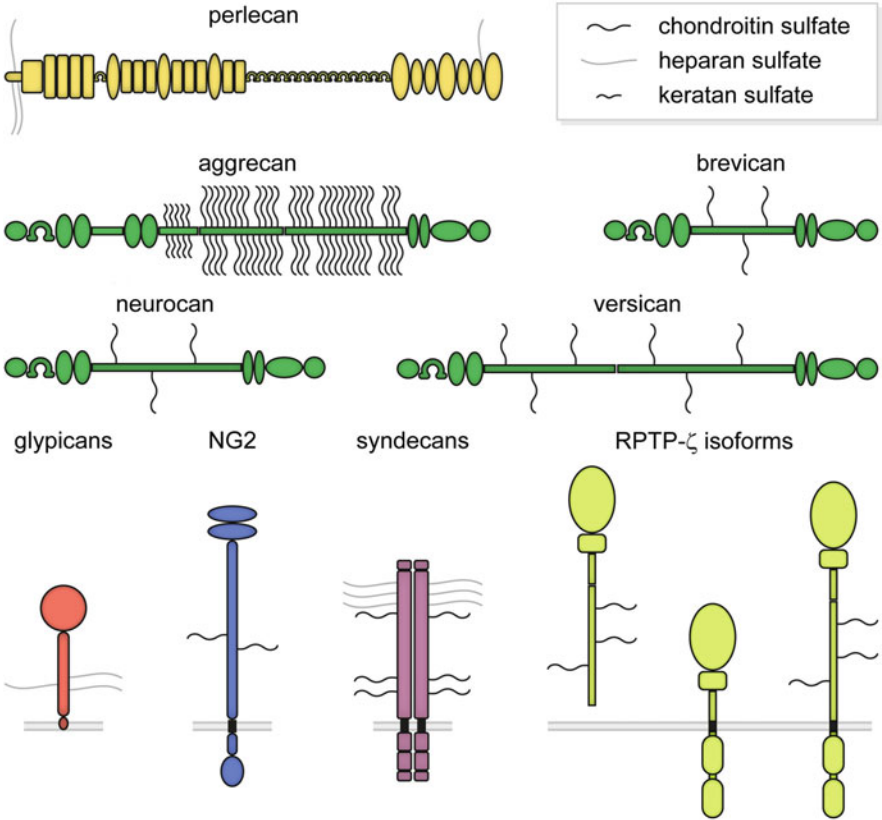

Fig. 8.1 Important proteoglycans of the CNS. Depicted are the secreted proteoglycans perlecan and the lectican family comprising aggrecan, brevican, neurocan, and versican. Membrane-associated proteoglycans are glypicans, NG2/CSPG4, syndecans, and two isoforms of the RPTP-ζ family. All molecules have a modular structure, a typical feature of extracellular matrix molecules. Potential sites for glycosaminoglycan modifications are indicated by black and gray lines, whereas glycosylation with shorter, branched glycans is omitted in the scheme

as well as distant physiological and pathophysiological signals and serve as "entities of action" (Scadden 2006). Furthermore, niches harbor a specific set of ECM compounds that are enriched in the interstitial space (Faissner and Reinhard 2015; Kazanis and ffrench-Constant 2011; Brizzi et al. 2012). In the adult mouse CNS two neurogenic regions are generally accepted, the subependymal zone of the lateral ventricle and the subgranular zone of the dentate gyrus of the hippocampus (Fig. 8.2) (Ihrie and Alvarez-Buylla 2011; Ming and Song 2005; Ma et al. 2005; Llorens-Bobadilla and Martin-Villalba 2017). The situation in the human brain is still under debate, with reports of newly formed neurons found in the striatum and inconsistent results for the aging adult hippocampus (Sorrells et al. 2018; Boldrini et al. 2018; Ernst et al. 2014).

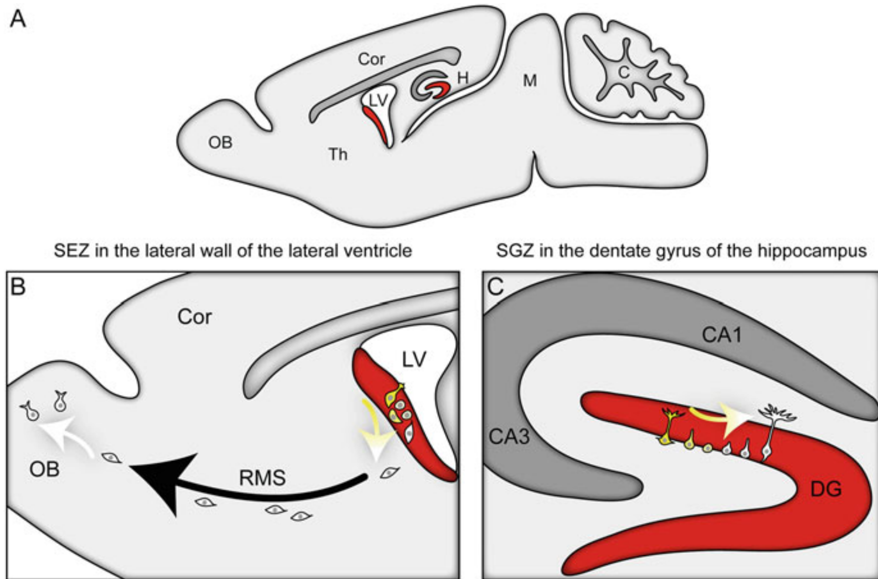

Fig. 8.2 Adult neural stem cell niches in the mouse brain. (**a**) Adult neurogenesis is observed in the subependymal zone (SEZ) of the lateral wall of the lateral ventricles and in the subgranular zone (SGZ) of the dentate gyrus region in the hippocampus (highlighted in red). (**b**) In the SEZ, neural stem cells (yellow) start to differentiate and migrate as neuroblasts via the rostral migratory stream to the olfactory bulb, where they terminally differentiate into neurons. (**c**) Adult neural stem cells in the SGZ give rise to neurons that integrate into the hippocampal network. White arrows indicate differentiation of adult neural stem cells, the black arrow in **b** indicates the direction of cells migrating via the rostral migratory stream. Cor, cortex; CA1, 3, cornu ammonis 1,3; DG, dentate gyrus; H, hippocampus; LV, lateral ventricle; M, midbrain; OB, olfactory bulb; RMS, rostral migratory stream; Th, thalamus

8.4 ECM of the Niche

Among the first ECM constituents revealed in the niche microenvironment of the adult CNS were the glycoprotein tenascin-C (Tnc) and a CSPG called DSD-1-PG, a mouse homolog of phosphacan (Gates et al. 1995; Steindler et al. 1996; Garwood et al. 1999; Faissner et al. 2017). Tnc has subsequently been found in various stem cell niches and can be considered as a model for glycoproteins of NSC-niches (Chiquet-Ehrismann et al. 2014; Faissner et al. 2017). Transcriptomes obtained from the adult SEZ and the oSVZ have confirmed the prevalence of selected ECM genes in neurogenic niches and indicated significant differences in expression patterns between the inner and the outer subventricular zones in the human embryonic brain. These findings corroborate the interpretation that the neural matrisome is crucial for the proliferation and self-renewal of NSCs in the developing human neocortex. In that context, ECM molecules of interest included tenascins, collagens, laminins, their respective integrin ECM receptors, and proteoglycans (Pollen et al.

2015; Fietz et al. 2012; LaMonica et al. 2012). In addition, the glycoprotein reelin, which controls the layering of cortical neurons, and its lipoprotein family VLDLR and ApoER2 receptors have been found involved in the regulation of neurogenesis in the dentate gyrus of the hippocampus (Zhao et al. 2007; Frotscher 1998, 2010; Drakew et al. 2002). Three-dimensional organoids derived from pluripotent stem cells represent new important tools to decipher the role of the ECM microenvironment of NSCs and its impact on neurodegenerative diseases, as they allow to study human cells in general, and in particular patient-derived cells (Yan et al. 2019).

In this chapter, we will discuss in detail the proteoglycans that emerge as an important support system for a large range of signaling processes (Iozzo and Schaefer 2015). Proteoglycans are classified according to their carbohydrate GAG-chains and within these general categories, subgroups can be subdivided according to the primary sequence of the glycoprotein cores (Bandtlow and Zimmermann 2000; Iozzo and Schaefer 2015).

8.5 The GAG-Chains of Proteoglycans

In general terms, glycosaminoglycan chains consist of repeating disaccharide units that are linked to the core protein via a common GAG-protein linkage region (Fig. 8.3). The linkage region is composed of a xylose covalently linked to serine of the proteoglycan core protein, followed by two galactose and one glucuronic acid moiety. The synthesis of this attachment site requires specific xylosyl- and galactosyl transferases (Mizumoto et al. 2015b). To this docking site, an *N*-acetylgalactosamine and an *N*-acetylglucosamine are added in case of a chondroitin sulfate (CS) and a heparan sulfate (HS) polymer chain, respectively. These carbohydrates form the starting point of the glycosaminoglycan polymers built of repeating disaccharide subunits.

8.6 Heparan Sulfate GAGs

HS is a polymer of repeating carbohydrate dimers that consist of *N*-acetylglucosamine and glucuronic acid that can be sulfated at the C2-, C3-, and C6-positions and at the C2-position, respectively (Fig. 8.3). The HS-polymerase enzymes Ext1 and Ext2 regulate the elongation of the chain. Modifications can be introduced by epimerization of *N*-acetylglucosamine and by the attachment of sulfate groups to distinct positions in the polymer (Sarrazin et al. 2011). The sulfation of HS generates charged microdomains that serve as docking sites for specific proteins such as fibroblast growth factor 2 (FGF-2), platelet-derived growth factor (PDGF), or anti-thrombin (Sarrazin et al. 2011). The biological significance of the HS-GAGs is highlighted by studies using a conditional knockout of the

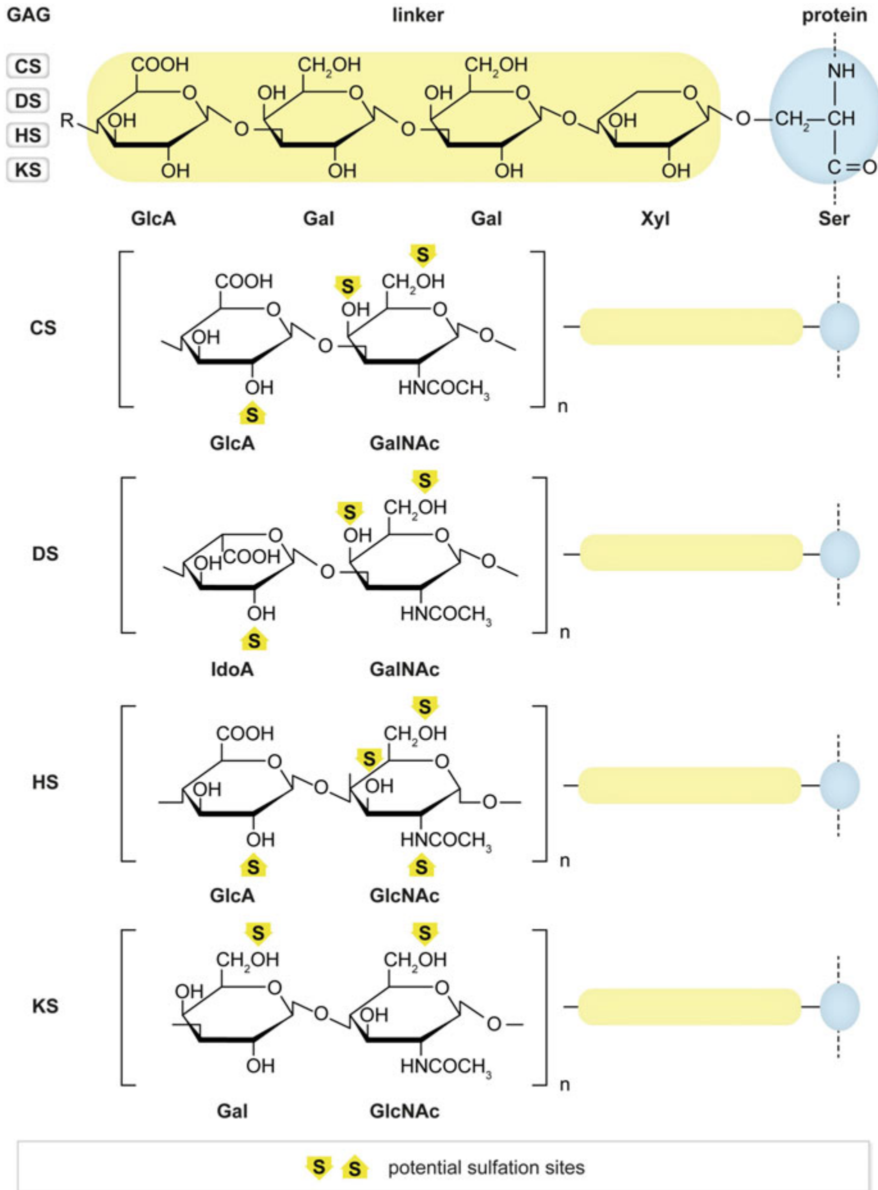

Fig. 8.3 Structure of proteoglycans. Proteoglycans (PGs) are composed of a core protein and at least one glycosaminoglycan (GAG) chain. GAGs consist of repetitive disaccharide units that are covalently bound to a serine (Ser) via a conserved linker tetrasaccharide. According to the disaccharide unit composition, chondroitin sulfate (CS), dermatan sulfate (DS), heparan sulfate (HS), and keratan sulfate (KS) can be distinguished. Sulfation can occur at specific sites (yellow arrows), but sulfation patterns differ depending on the context. CS can be converted to DS by the enzyme DS-epimerase, leading to hybrid chains containing CS and DS units. Gal, galactose; GalNAc, *N*-acetylgalactosamine; Glc, glucose; GlcA, glucuronic acid; GlcNAc, *N*-acetylglucosamine; IdoA, iduronic acid; Xyl, xylose

glycosyltransferase Ext1, which is essential for heparan sulfate synthesis (Irie et al. 2012; Yamaguchi et al. 2010).

8.7 Heparan Sulfate Proteoglycans

Families of HSPGs have been distinguished on the basis of the primary amino acid sequence of the protein core. HSPGs are found in almost all mammalian cells. They are membrane-associated (glypicans, syndecans) or enriched in the interstitial space (perlecan, collagen type XVIII, or agrin) (Iozzo and Schaefer 2015). In the neural stem cell niche, both the syndecans (SDC), which contain a transmembrane domain, and the glypicans (GPC), which interact with membranes via a GPI-anchor, have been reported (Yamaguchi et al. 2010). Thus, the neuroepithelial stem cell expresses SDC1 and SDC4, as well as GPC1 and GPC4. The expression patterns change when the neural stem cell evolves to radial glia and engages into distinct lineage differentiation pathways. For example, the immature astrocyte expresses SDC4, GPC1, GPC4, and GPC6 whereas the oligodendrocytes expose SDC2 and SDC4 (reviewed by Yu et al. 2017). Interestingly, astrocyte-derived GPC4 and GPC6 play an important role in the maturation of excitatory synapses, a differentiation step that is strongly controlled by neuron–glia interactions (Allen et al. 2012; Freeman 2005, 2010). The differential pattern of HSPG expression may reveal important regulatory roles in the stem cell compartment. In this regard, it is of note that HSPGs exert important functions, which include ligand–receptor clustering, storage, and presentation of morphogens such as sonic hedgehog and Wnt proteins, or the cytokine FGF-2 (summarized in Sarrazin et al. 2011), and integrin function (Stenzel et al. 2014). The HSPGs immobilize specific cytokines or signaling molecules via particular recognition sequences in the HS-chains and expose these to the cognate receptors. A prominent example is the interference of GPC with FGF-2 that affects binding to the FGF receptor (FGFR) and regulates FGF-2-dependent stem cell proliferation (Hagihara et al. 2000; Jen et al. 2009), or the presentation of Wnt proteins by SDC1 to the frizzled receptor and other interactions (Bishop et al. 2007; Song et al. 2005; Wang et al. 2012). In cancer cells, HSPGs also intervene in the Notch-delta signaling pathway that is essential for radial glia maintenance (Yanagisawa and Yu 2007; Androutsellis-Theotokis et al. 2006; Ibrahim et al. 2017).

Binding sites in the HS-chains depend on sulfation and the idea has been discussed that particular sulfation codes of HS contribute to the control of development and plasticity (Kraushaar et al. 2013; Poulain and Yost 2015; Sarrazin et al. 2011). When bifurcating into the differentiation pathway of neurons, neuroepithelial cells shift the 6-O-sulfation profile and HS-chain length. These modifications correlated with a differential responsiveness toward FGF-1 and FGF-2 signaling (Brickman et al. 1998a, b). Changes in N-sulfation, 3-O-sulfation, and 6-O-sulfation have been noted along with stem cell differentiation. The reduction of sulfation during in vitro neural stem cell differentiation by the addition of the sulfation inhibitor sodium chlorate modifies the relative proportion of immature neurons

derived from NSCs and blocks their further differentiation (Karus et al. 2012; Xiong et al. 2014).

Beyond the support of ligand–receptor interactions, HSPGs play also a role in the activation of integrins and cell adhesion molecules. Thereby, HSPGs are implicated in cell–substrate interactions as well as motility and influence endocytosis. These processes represent important steps during CNS histogenesis, where HSPGs are involved in the regulation of progenitor proliferation and in axon growth and guidance (Maeda 2015).

By virtue of their binding capacities, HSPGs that are shed into the pericellular environment serve as storage sites for various ligands that can be deposited into pericellular interactomes of the matrisome (Sarrazin et al. 2011). The HSPG perlecan is an essential component of basement membranes and contributes to the formation of tissue barriers (Sarrazin et al. 2011). In the adult, fractones, assemblies of laminin glycoproteins, nidogen, collagen IV, and HSPGs, have been detected in the SEZ (Kerever et al. 2007). Fractones capture the cytokine FGF-2, which modulates proliferation and differentiation of NSCs in the niche. This effect depends on the HSPG perlecan (Kerever et al. 2014). A reduction of 6-O-sulfation of heparan sulfate in the aging neurogenic niche seems to be critical for reduced FGF-2 signaling (Yamada et al. 2017). Treatment of mouse embryonic stem cells with the endoglucuronidase heparanase resulted in an enhanced differentiation to oligodendrocytes (Xiong et al. 2017).

8.8 Chondroitin Sulfate GAGs and CSPGs

CS-GAGs are also composed of long chains of repeating dimers of carbohydrates, namely *N*-acetylgalactosamine and glucuronic acid. These chains are bound to the common GAG-protein linkage region and extended by CS-synthases. Biosynthesis and maturation of the CS-polymers occur in the endoplasmic reticulum and the Golgi apparatus (Sugahara and Mikami 2007; Sugahara et al. 2003). CS can be converted to dermatan sulfate (DS) by DS-epimerase enzymes that modify the stereochemistry of glucuronic acid and convert it to iduronic acid. Hybrid chains with CS and DS units are the result. A further important modification of CS-compounds is based on sulfation. Specific sulfotransferases attach sulfate groups to the carbohydrates, creating units that are distinguished by the position of the sulfate group in the dimers. Thus, CS-A is characterized by a sulfate group at the fourth carbon (C4) of *N*-acetylgalactosamine (GlcA-GalNac(4S), A unit), whereas CS-C possesses the sulfate at C6 (GlcA-GalNac(6S), C unit). In CS-GAGs, also units have been distinguished that contain two sulfate groups, among them the CS-D and CS-E units (Sugahara and Mikami 2007; Karamanos et al. 2018). The CS-chains can attain considerable lengths with more than 100 units, where the individual units can differ. Therefrom a tremendous diversity of CS-GAGs emerges that is characterized by particular spatial arrangements of charges, in particular sulfates, along the GAG chains (Purushothaman et al. 2012). Mounting evidence suggests that these

structural features create docking domains with specific recognition sites for distinct protein ligands (Mizumoto et al. 2015a).

8.9 Discrete CS-GAG Structures

8.9.1 The DSD-1-Epitope

The DSD-1-epitope represents a well-studied example of a sulfation-based antigenic CS structure (Fig. 8.4a, b). It was originally discovered as the binding site of the monoclonal antibody 473HD, which recognizes the surface of immature glial cells. Further characterization of the epitope revealed that it is based on chondroitinase ABC (ChABC)-sensitive CS-type GAG-structures (Faissner et al. 1994). The DSD-1-epitope proved resistant to chondroitinase ACII, but not ACI, suggesting that it contains dermatan sulfate and hence depends on iduronic acid ("dermatan sulfate-dependent"). Because in vitro evidence suggested that the DSD-1-epitope is involved in neurite outgrowth promotion of embryonic day 18 hippocampal neurons, the structural characterization was carried further. It could be shown that the epitope

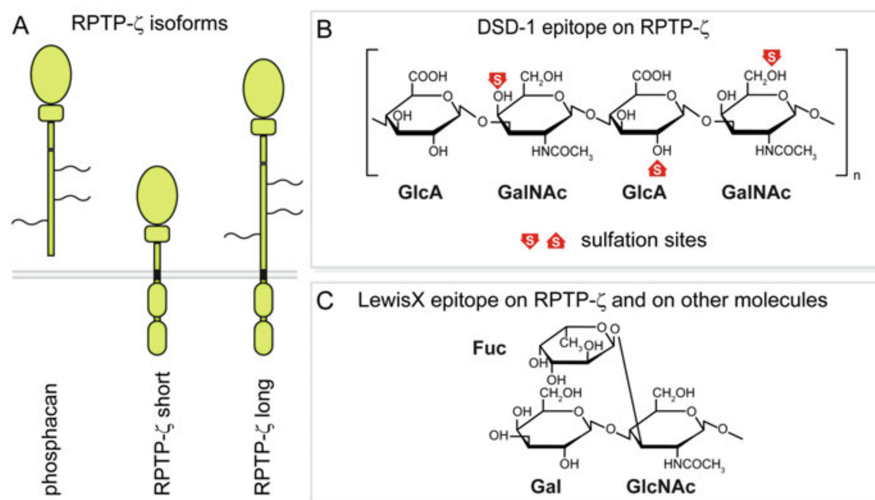

Fig. 8.4 Distinct glycoepitopes are associated with neural stem cells. (**a**) The family of receptor protein tyrosine phosphatase (RPTP)-ζ comprises three isoforms, with one secreted (phosphacan) and two transmembrane forms (RPTP-ζ long and RPTP-ζ short). (**b**) Phosphacan and RPTP-ζ long can carry chondroitin sulfate chains with a specific motif, called DSD-1 epitope. It is defined by a distinct sulfation pattern, as indicated by the red arrows. The epitope has been identified on neural stem cells and its blockage affects neurosphere formation in vitro. (**c**) The LewisX trisaccharide motif can be attached to, but is not limited to proteoglycans like RPTP-ζ. It is expressed on radial glia and neural stem cells. Fuc, fucose; Gal, galactose; GalNAc, *N*-acetylgalactosamine; GlcA, glucuronic acid; GlcNAc, *N*-acetylglucosamine

is enriched in CS-D and thereby integrates highly sulfated CS-motifs (Clement et al. 1998; Hikino et al. 2003; Ito et al. 2005). Interestingly, the monoclonal antibody (Mab) 473HD does not recognize CS-E, although the latter GAG also comprises the E-units with two sulfate moieties, strongly supporting the notion that sulfation creates docking sites for proteins based on the arrangement of sulfate groups, rather than solely on charge (Clement et al. 1999). The CS-motifs can serve as docking sites for various ligands and also as recognition signals for specific receptors, e.g., of the receptor protein tyrosine phosphatase (RPTP) family and intervene in signaling processes in many ways (Shen et al. 2009; Yu et al. 2018; Purushothaman et al. 2012). Participation in cell signaling processes may be of importance for the biology of CSPGs in the neural stem cell niche. There, a variety of CSPGs has been detected (Kabos et al. 2004). Using the Mab 473HD it could be shown that the DSD-1-epitope is specifically enriched on the surface of radial glia NSCs (von Holst et al. 2006). Both ChABC digestion of NSCs and the blockade of the epitope by Mab 473HD reduced the proliferation rate of the NSCs in an in vitro neurosphere culture model (von Holst et al. 2006). Important insights into the functions of CS-GAGs have been obtained by the application of this bacterial-derived enzyme to the developing or mature CNS, revealing important roles in memory function in the hippocampus and in plasticity after injury in the spinal cord (Dityatev et al. 2010a; Fawcett 2015). The application of ChABC both in vitro and in vivo, therefore, is an established strategy to challenge the functional role of CSPGs in biological systems including the neural stem cell niche (Sirko et al. 2010). Injection of ChABC into the ventricular space of E13 mouse embryos resulted in reduced proliferation of NSCs in vivo (Sirko et al. 2007). The treatment also favored the development of astrocytes and retarded neurogenesis in the in vitro neurosphere cell culture assay (Sirko et al. 2007). This process most probably involved the FGF-2-dependent signaling processes in NSCs, suggesting that CS-GAGs are also participating in FGF-2 signaling. This conclusion is consistent with a series of observations implicating CS in ligand-dependent signaling mechanisms (Djerbal et al. 2017). Moreover, in agreement with the concept that the sulfation pattern of the CS-GAGs is critical for the generation of ligand docking sites, the relevant sulfotransferases have been detected in the neural stem cell niche of embryonic and juvenile mice, both in vivo and also in neurosphere culture assays in vitro (Akita et al. 2008). Suppression of sulfation by sodium chlorate in spinal cord-derived neurospheres resulted in a dose-dependent decrease in the formation of neurospheres, an altered cell cycle progression, enhanced neurogenesis, and delayed neuronal maturation (Karus et al. 2012). This is in line with the abovementioned observation that a reduction of 6-O-sulfation of heparin sulfate in the aging neurogenic niche seems to be critical for reduced FGF-2 signaling (Yamada et al. 2017). Besides its important role in the developing organism, the DSD-1-epitope has been shown to be re-expressed by reactive astrocytes with immature properties in the adult brain after lesion, indicating a stem cell-associated function of the carbohydrate motif also in this context (Roll et al. 2012; Sirko et al. 2009).

8.9.2 RPTP-ζ and Phosphacan

Glycosaminoglycans with the exception of hyaluronic acid are covalently coupled to proteoglycan core proteins. The DSD-1-epitope is expressed by a CSPG termed DSD-1-PG that was identified as the mouse homolog of rat phosphacan using biochemical purification and expression cloning strategies (Garwood et al. 1999, 2001; Faissner et al. 1994). The CSPG phosphacan is an isoform of receptor protein tyrosine phosphatase-zeta (RPTP-ζ), a transmembrane tyrosine phosphatase receptor. Tyrosine phosphatases antagonize tyrosine kinase-dependent signaling pathways and most probably exert modulating functions in a variety of important signaling processes (Tonks 2013). Structurally, it is possible to distinguish 21 transmembrane receptors and 16 non-transmembrane cytoplasmic types that all harbor the characteristic tyrosine phosphatase domain. RPTP-ζ occurs as a large transmembrane receptor with a large ectodomain that corresponds to phosphacan. A small receptor variant that is devoid of the ectodomain has also been described (Fig. 8.1). A short version of the secreted phosphacan has been reported as fourth protein variant that may either be the result of alternative splicing or a product of proteolytic cleavage (Garwood et al. 2003; Chow et al. 2008). Phosphacan is a ligand of the ECM glycoprotein tenascin-C and highly expressed in the adult stem cell niche (Gates et al. 1995; Faissner et al. 2006). Moreover, the large and the short receptor variants have been detected in NSCs and most probably regulate important biological functions in the stem cell compartment (Garwood et al. 2001; Faissner et al. 2017; Lamprianou and Harroch 2006). Thus, RPTP-ζ has been characterized as a receptor of the cytokine pleiotrophin (Mohebiany et al. 2013) and interleukin-34 (Nandi et al. 2013). Pleiotrophin signaling is involved in oligodendrocyte differentiation and along these lines the digestion of oligodendrocyte precursors by ChABC results in changes of OPC proliferation and differentiation (Kuboyama et al. 2016; Karus et al. 2016).

8.9.3 Lecticans

The CSPGs of the lectican family, aggrecan, versican, neurocan, and brevican, constitute an important subfamily of the CNS matrix (Bandtlow and Zimmermann 2000). Lecticans exert roles in neuronal plasticity and in axon guidance and repair following injury of the nervous system (Bekku et al. 2009; Kwok et al. 2011). During embryonic development, CSPGs are involved in neuron migration, axon guidance, the regulation of glial cells and synapse formation (Bandtlow and Zimmermann 2000; Heikkinen et al. 2014; Maeda 2015; Maeda et al. 2011; Meyer-Puttlitz et al. 1996; Wiese et al. 2012). CSPGs of the lectican family structurally are composed of a core protein and covalently linked non-branched GAG chains. Each of these CSPGs comprises (1) an N-terminal G1 domain including an immunoglobulin-like domain (Ig-domain) in conjunction with proteoglycan tandem

repeats domains involved in binding hyaluronic acid, (2) a CS-GAG attachment region, (3) a C-terminal G3 domain containing epidermal growth factor- (EGF-) like domains, a lectin-like domain, and a complement regulatory protein-like domain (Avram et al. 2014; Zimmermann and Dours-Zimmermann 2008). Lecticans can interact with hyaluronic acid on the one hand and with glycoproteins of the ECM on the other hand, thereby contributing to the condensation of large interactomes such as the ECM of the stem cell niche, or perineuronal nets (Maeda 2015; Dzyubenko et al. 2016; Geissler et al. 2013). The lectican aggrecan is present in PNNs and used as a marker in many studies (Sorg et al. 2016). Versican is expressed in oligodendrocytes and occurs in four isoforms (V0–V3; Zimmermann and Dours-Zimmermann (2008). Neurospheres create an environment rich in CSPGs containing phosphacan and the lecticans that can be traced in the culture medium (Wade et al. 2014; Kabos et al. 2004; Ida et al. 2006).

8.9.4 NG2/CSPG4

NG2 (also designated CSPG4) was originally discovered as a neuron-glia-based antigen with prominent expression in the gray matter of the cerebellum (Stallcup et al. 1983). Biochemical and localization studies have revealed that NG2 is a CSPG with strong expression in the oligodendrocyte lineage and involved in the regulation of oligodendrocyte precursor (OPC) proliferation and migration (Nishiyama et al. 2009; Trotter et al. 2010). NG2 regulates the directional migration of OPCs via the activation of a Rho-GTPase-linked signaling pathway (Binamé et al. 2013). In the context of cell motility, Ca^{2+} signaling pathways are activated (Haberlandt et al. 2011). NG2 cells abound in the gray matter of the adult CNS and it has been proposed that they may represent a glial lineage in their own right (Nishiyama et al. 2009). As a particular feature, these glial cells can receive synapses from neurons and thereby may modulate the activity of neuronal circuits (Gallo et al. 2008; Etxeberria et al. 2010; Sakry et al. 2011; Dimou and Gallo 2015). The question has been addressed whether NG2-positive glia is confined to the glial lineage or may develop into other neural cell types, thus displaying progenitor or even stem cell properties. A study based on a genetic labeling strategy concluded that the overwhelming majority of NG2 glia differentiates into oligodendrocytes during development (Huang et al. 2014). However, the situation may be quite different after lesion because upon stab wounding fate-mapped NG2 cells could be converted to neurons by retroviral expression of the transcription factors Ascl1 and Sox2, or even Sox2 alone. These neurons matured and developed synaptic contacts, clearly pointing to their functional potential. Neurogenesis from NG2 cells could not be obtained in the absence of a lesion, however (Heinrich et al. 2014). These observations could indicate a broader developmental potential of NG2 glia under certain circumstances.

8.10 Complex Glycans

Not limited to proteoglycans are glycan moieties of the LewisX (LeX; Fig. 8.4c) subfamily. They are expressed on the surface of radial glia and neural stem cells (Hennen et al. 2011, 2013). Using the newly generated monoclonal antibodies 5750^{LeX} and 487^{LeX}, specific LeX-type glycans could be located to the core glycoproteins of the CSPG RPTP-ζ and its isoform phosphacan and also to the ECM glycoprotein tenascin-C (Hennen et al. 2011). Furthermore, the multimodular membrane receptor lipoprotein receptor-related protein 1 (LRP1) was identified as a LeX-exposing membrane protein of neural stem cells that can be used for the sorting of NSCs. The elimination of LRP1 from NSCs by Cre-mediated recombination impairs oligodendrogenesis and favors the differentiation of astrocytes (Safina et al. 2016).

8.11 Glioblastoma Stem Cells

Glioblastoma multiforme (GBM) is the highest-grade glioma (grade IV) tumor and belongs to the most malignant brain tumors (Wen and Kesari 2008; Stupp et al. 2017). No effective therapy is available for the treatment of GBM and survival times after diagnosis only rarely reach beyond 1 year. Recently, the concept has been developed that recurrence of GBM could be linked to the existence of cancer stem cells (CSCs) (Kreso and Dick 2014; Lapidot et al. 1994; Clarke et al. 2006; Reya et al. 2001; Magee et al. 2012). Glioblastoma shows a considerable cellular heterogeneity. It has been proposed that cancer stem cells are at the origin of glioblastoma tumors that are referred to as glioblastoma stem cells (GSCs) (Lathia et al. 2015). GSCs differ from their progeny with regard to their transcriptome, the metabolic and epigenetic state (Gimple et al. 2019). These cells reciprocally interact and thereby promote malignant progression (Wang et al. 2018).

In current GBM clinical treatment protocols, the chemotherapeutic drug temozolomide (TMZ) is applied that targets a fast dividing subpopulation of brain tumor cells. In contrast, the brain tumor stem cells are quiescent, slow dividing, resistant to TMZ and irradiation treatment and get activated during tumor relapse (Chen et al. 2012; Zhu et al. 2014; Bao et al. 2006). It is thought that also the CSCs of the CNS, in particular the GSCs, reside in specialized niches. These niches are rich in ECM compounds that show similarities to the niche environment of the developing and adult CNS (Brösicke and Faissner 2015; Reinhard et al. 2016). GBM is a highly invasive solid tumor and penetrates the CNS by expanding along myelin-rich pathways, leading to the colonization of both hemispheres. The GBM-related ECM is believed to support the migratory activity and invasiveness of the tumor (Dunn et al. 2012; Clark and Vignjevic 2015). Thus, the ECM in the microenvironment regulates invasiveness of glioblastoma (Lathia et al. 2011; Kim et al. 2018). The ECM is amenable to modifications by monoclonal antibodies or small molecule

drugs, and therefore may represent an interesting therapeutic target. The relevance and efficacy of ECM-related treatments towards GBM still remain, however, to be demonstrated.

8.12 Proteoglycans in GBM

As discussed previously, proteoglycans are pivotal constituents of the stem cell niche microenvironment where they play important roles in signaling processes. Therefore, proteoglycans most probably play analogous roles with regard to cancer stem cells in their niches (Wade et al. 2013). The hyaluronic acid receptor CD44 is highly expressed in GBM, indicative of the presence of a hyaluronic acid-rich ECM in the GBM microenvironment (Mooney et al. 2016; Brown et al. 2017). The interaction of GBM cells with hyaluronic acid via the CD44-receptor plays an important role in tuning invasiveness (Kim and Kumar 2014).

Hyaluronic acid is a prominent ligand of the lectican family CSPGs that could be part of a GBM-based ECM environment. A high expression of CSPGs seems to correlate with low invasiveness of the tumor, presumably due to the inhibitory and anti-adhesive properties of CSPGs for neural cell types (Silver et al. 2013). Consistent with this assessment, the V1 isoform of the lectican versican has been detected in GBM. Treatment of GBM with a versican V1 siRNA resulted in reduced proliferation of the tumor cells (Onken et al. 2014).

Furthermore, the CSPG phosphacan, an isoform of the large RPTP-ζ receptor, is expressed in OPCs and downregulated upon differentiation to oligodendrocytes (Karus et al. 2016). As it is also strongly prevalent in NSCs, its association with GBM is not unexpected. There it may represent a promising target for the treatment of the disease (Fujikawa et al. 2017; Zeng et al. 2017). The downregulation of RPTP-ζ by siRNA interfered with GBM growth both in vitro and in vivo (Ulbricht et al. 2006). Also in the context of CSCs the regulatory loop involving pleiotrophin and RPTP-ζ may thus play a role and drive the expansion of the tumor (Shi et al. 2017).

NG2/CSPG4 is expressed by glioblastoma cells and its level of expression correlates with the degree of malignancy (Tsidulko et al. 2017; Lama et al. 2016). The NG2-positive cells are characterized by active proliferation and seem to be resistant against radiation therapy, consistent with CSC properties in GBM (Al-Mayhani et al. 2011; Svendsen et al. 2011). Based on these observations, attempts using specific Mabs have been made to validate NG2/CSPG4 as a therapeutic target (Poli et al. 2013). Summarizing these reports there is mounting evidence that the association of the neural stem cell niche with distinct proteoglycans also occurs in the niche environment of CSCs in particularly malignant brain tumors. Targeting this group of molecules of the neural matrisome may lead to new therapeutic avenues.

8.13 Concluding Remarks

Proteoglycans are potent regulators of the cell fate and of cell functions in the developing, but also in the adult organism. A better understanding of these important constituents of the neural stem cell niche can contribute to advances in stem cell biology, which is also of interest in the light of stem cell-based therapies in the future. In cancers such as glioblastoma, proteoglycans represent a promising therapeutic target to attack tumors by manipulating the cells' microenvironment.

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Conflict of Interest Both authors declare that they have no conflict of interests.

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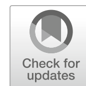
Chapter 9
Heparan Sulfate in Normal and Cancer Stem Cells of the Brain



Anqi Xiong, Ananya Roy, Argyris Spyrou, and Karin Forsberg-Nilsson

Abstract Proteoglycans are key molecules in signaling, both during brain development and in malignant brain tumor formation, where cancer cells mimic, or co-opt, normal developmental programs. This chapter focuses on the role of heparan sulfate proteoglycans (HSPGs) in these processes. HSPGs are composed of a core protein with attached, heavily sulfated, polysaccharide side chains, and they are ubiquitously present on cell surfaces and in the extracellular space where they serve both as structural components and regulators of a multitude of cellular activities. HSPGs are critically involved in mammalian development, and perturbations of pathways regulated by HSPGs play major roles in human diseases. Neural stem cell programs sustain populations of stem cells that initially give rise to neural progenitors with high proliferative capacity that eventually differentiate to mature cells of the nervous system. HSPGs act as coreceptors for a wide variety of signaling pathways during these processes. Accumulated mutations in neural stem cells can cause failure to perform terminal differentiation or the inability to restrict progenitor proliferation and lead to brain tumor development. The same signaling mechanisms that promote self-renewal of neural stem cells thus also support cancer stem cells, and HSPGs are integral facilitators of brain tumor development and progression.

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9.1 Neural Stem Cells and Brain Tumor Stem Cells

In the central nervous system (CNS), the stem cells that give rise to neurons, astrocytes, and oligodendrocytes are called neural stem cells (NSCs). In the earliest stage of neural development, a single layer of neuroepithelial stem cells, the neural plate, invaginates to form the neural tube, a process called neurulation. Next, the neural tube's anterior part balloons out and forms the three primary vesicles, namely, the prosencephalon (forebrain), mesencephalon (midbrain), and rhombencephalon (hindbrain), while the posterior neural tube develops into the spinal cord. Subsequently, these three vesicles will become five (termed the telencephalon, diencephalon, mesencephalon, metencephalon, and myelencephalon), which later forms the different structures of the new brain.

The fate-restricted progenitor cell descendants of neural stem cells, i.e., neurons, astrocytes, and oligodendrocytes develop at different times throughout brain development. In the mouse brain, neurulation commences around embryonic day (E) 8.5. At this time, neuroepithelial cells which form the neural plate are bona fide NSCs. These undergo so-called symmetric division to enlarge the number of stem cells (McConnell 1995; Rakic 1995). When neurogenesis begins at approximately E9–10 in the mouse, the neuroepithelial cells begin to transform into radial glial cells. The latter maintain certain neuroepithelial cell properties, including the expression of nestin, apical-basal polarity, and interkinetic nuclear migration (Chenn et al. 1998; Hartfuss et al. 2001). At the same time, they undergo morphological changes, including the lengthening of the pial-directed radial processes. These cells also start to express markers, such as astrocyte-specific glutamate transporter (GLAST) (Shibata et al. 1997) and brain lipid-binding protein (BLBP) (Feng et al. 1994). Radial glial cells serve as neural progenitors throughout the CNS (Anthony et al. 2004; Malatesta et al. 2003). In contrast to neuroepithelial cells, radial glial cells undergo asymmetric cell division to self-renew and produce a daughter cell that is a more restricted intermediate progenitor cell (Haubensak et al. 2004; Noctor et al. 2004). These intermediate progenitor cells eventually give rise to neurons and later to astrocytes and oligodendrocytes. The radial glial cells are highly heterogeneous in terms of the progeny they produce. This diversity is established by differences in sets of transcription factors they express in response to the microenvironment (Flames et al. 2007; Guillemot 2007; Long et al. 2009; Puelles and Rubenstein 2003).

NSCs are also present in restricted regions of adult mouse brains. The subventricular zone (SVZ) of the lateral ventricles is considered the prominent proliferation region (Puelles and Rubenstein 2003) (Fig. 9.1). A subset of subventricular cells that express the glial acidic fibrillary protein (GFAP) in this region has been identified as adult NSCs (Doetsch et al. 1997). They have been termed B cells and are relatively quiescent. B cells, in their turn, can give rise to cycling progenitor cells, so-called C cells (Doetsch et al. 1999), with the capacity to differentiate into A cells (immature neuroblasts) that by movement along the rostral-migratory stream find their way to the olfactory bulb, where they differentiate into

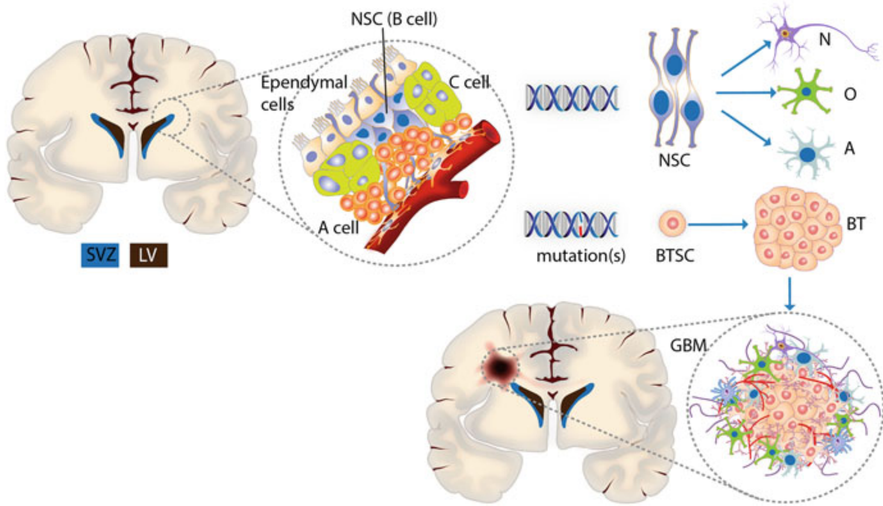

Fig. 9.1 Adult neural stem cells and brain tumor stem cells. The subventricular zone (SVZ) of the lateral ventricle (LV) is a major neurogenic niche (upper dotted circle) in the adult human brain. Here, adult neural stem cells (NSC), also called B cells, reside close to the ependymal cells that line the ventricle. B cells are relatively quiescent and will, upon differentiation, give rise to cycling progenitor cells, so-called C cells that have the capacity to differentiate into A cells (immature neuroblasts). B cells can also give rise to glia. When B cells are hit by mutations in growth regulatory pathways, it is believed that they can give rise to brain tumor stem cells (BTSC), often after a long latency period, and initiate the formation of a brain tumor (BT). Glioblastoma (GBM) is thought to arise through the malignant transformation of NSCs. The GBM tumor microenvironment (lower dotted circled) is very complex and contains infiltrating non-tumor cells, a highly proliferating and abnormal vasculature, and dynamic alterations of the extracellular matrix that supports the malignant phenotype

interneurons (Belluzzi et al. 2003; Carleton et al. 2003). Furthermore, B cells also give rise to a subpopulation of oligodendrocytes (Menn et al. 2006). Another major region for NSCs in the adult brain is the dentate gyrus (DG) of the hippocampus. These NSCs are located in the subgranular zone (SGZ) and can differentiate into neurons and astrocytes (Suh et al. 2007). Neurogenesis in this region has been implicated in learning and memory in rats (Shors et al. 2002), and aberrant neurogenesis in this region has been linked to stroke, neuroinflammation, and epilepsy (Zhao et al. 2008). The adult neurogenesis in humans differs from that of rodents, but adult human neural stem cells were identified over 20 years ago (Eriksson et al. 1998). Despite some controversy around its extent and significance, it is clear that human neurogenesis exists, and plays important roles in neural plasticity and at the same time contributes to cognition during the life of an adult, as summarized in Kempermann et al. (2018).

The concept of cancer stem cells (CSCs) describes a stem-cell-like cell that is believed to initiate tumor formation (reviewed in Clevers 2011). The term "cancer stem cell" is also used for those rare self-sustaining cells in a tumor with a resistance

to irradiation and chemotherapy, which the bulk of tumor cells lack (Magee et al. 2012). These CSCs, which can reside quiescently in the tissue and yet undergo division to seed a new tumor, are believed to be responsible for relapse after therapy. CSCs thus possess a unique capacity for growth and progression, a mechanism, which is self-governed and distinct from the usual regulatory controls of normal stem cells. The CSC hypothesis predicts that solid tumors have a hierarchical organization, where CSCs drive tumor maintenance and recurrence. Tumor expansion would thus be the result of unlimited ability for self-renewal by CSCs that are more resistant to chemotherapy and irradiation than the majority of tumor cells. Therefore, unless the CSCs are targeted, cancer treatment will not be successful.

In physiological homeostasis, the regulation of stem cell number in tissues is highly controlled since mutations affecting stem cells may result in uncontrolled proliferation and can trigger the development of cancer. It is now well established that concomitant with the role of NSCs in tissue development and repair, developmental stem cell programs are recapitulated in CSCs to support the development of tumors and sustain their malignancy, which has also been shown to be true in several cancers of the nervous system (Lathia et al. 2015) (Fig. 9.1). Therefore, the biology of NSCs is intertwined with that of brain tumor stem cells (BTSCs). Several investigators have described this concept in relation to malignant brain tumors, both in adults (Singh et al. 2003) and in childhood brain tumors (Hemmati et al. 2003). Over the last decade, attempts have been made to define the characteristics and markers of BTSCs (reviewed in Lathia et al. 2015), and recently, SVZ NSCs harboring driver mutations were shown to be the cell of origin for human glioblastoma (GBM) (Lee et al. 2018). The BTSC hypothesis has also been challenged by the concept of intrinsic plasticity driving tumor potential in a nonhierarchical manner (Dirkse et al. 2019). Oligodendrocyte progenitor cells (OPCs) have also been suggested as BTSCs for glioma, based on studies of genetically modified mice (Weiss et al. 2003; Lindberg et al. 2014) and the fact that OPCs constitute a large pool of proliferating cells in the adult brain (Nishiyama et al. 2009). Whether NSCs and OPCs give rise to different types of glioma and/or GBM remains to be elucidated.

NSCs exist in discrete compartments, also called niches, in the developing and adult brain (described above). These niches depend on microenvironmental cues and cell-cell interactions to support NSC proliferation and differentiation by integrating both local and distant signals. In the same way, the tumor microenvironment is a critical driver of heterogeneity, plasticity, and evolution within BTSC niches. For successful novel brain tumor therapies targeting BTSCs, a deeper understanding of BTSC biology concentrating on how they interact with the tumor microenvironment is needed. While substantial advances have been made to understand some of these compartments, such as the contribution of immune cells and brain tumor blood vessels (Quail and Joyce 2017), much less is known about how the network of extracellular matrix (ECM) molecules controls the availability of trophic factors and mechano-physical properties of the brain tumor microenvironment.

9.2 Introduction to the Extracellular Matrix in Neural Stem Cells and Brain Tumors

Studies of ECM function have led to an increased understanding of the biology of normal NSCs, as well as how malignant transformation of these cells can lead to CNS tumors. The ECM is a complex network of macromolecules, such as proteins, glycosaminoglycans (GAGs), glycoproteins, and proteoglycans. These are all assembled into organized structures, regulating proliferation, cell survival, differentiation to mature cell types, migration, and invasion. During brain development, ECM components are regulated in a well-coordinated manner to promote differentiation, neural cell migration, and axonal growth (Bandtlow and Zimmermann 2000), and in adulthood, the ECM maintains and controls functions such as synaptic plasticity and synaptic remodeling (Dityatev and Schachner 2003). For brain tumors, ECM molecules are emerging as important regulators in the tumor microenvironment, and their composition is often distinct from that of the normal brain. Understanding the ECM of brain tumors has been proposed as one of the recent challenges in the path to cure primary brain tumors (Aldape et al. 2019).

9.3 Extracellular Matrix Organization in the Brain

The ECM of the normal brain is distinct from that of other organs and stem cell niches, and consequently, the ECM of brain tumors is different from that of non-CNS tumors. Any tumor stroma outside the brain is usually rich in fibrillar collagens (Ruoslahti 1996), while in the CNS, GAGs, proteoglycans, and glycoproteins are the predominant constituents. ECM molecules, in addition to their structural and organizing functions, are fundamental for understanding brain tumor biology. They comprise 10–20% of the brain (Bignami et al. 1993) and not only provide structural support but also are an essential part of the neural signaling cascade. The adult brain ECM can be described in terms of three compartments of the neural interstitial matrix, i.e., (1) ECM molecules in the parenchyma, (2) the basement membrane ECM, and (3) the perineuronal nets. The ECM of the brain parenchyma consists mainly of hyaluronan and proteoglycan networks, which are produced intracellularly and then secreted into the extracellular space (Bignami et al. 1993), where they can assemble extracellular interlacing structures being either attach to the cell membrane or free (Barros et al. 2011). Other components include glycoproteins such as tenascins, reelin, chemotropic proteins like netrin and to a smaller extent, collagens, laminin, and fibronectin. The basement membrane surrounds the pial surface and forms a barrier between the vasculature and the parenchyma. It contains mainly collagen IV, laminins, fibronectin, dystroglycan, and heparan sulfate proteoglycans, e.g., in the form of perlecan (Itoh et al. 2011). Finally, the perineuronal nets are mesh-like structures of proteoglycans, tenascin R, and link proteins around neuronal cell bodies (Kwok et al. 2011). The role of perineuronal nets is to stabilize

synapses; therefore, these nets are important regulators of CNS plasticity (Wang and Fawcett 2012).

9.4 Heparan Sulfate Proteoglycans and Their Biosynthesis, Modification and Degradation

Heparan sulfate proteoglycans (HSPGs) are usually subdivided into three categories, depending on their location. These are membrane HSPGs, including syndecans and glycosylphosphatidylinositol-anchored (GPI) proteoglycans (glypicans), secreted HSPGs present in the ECM (perlecan, agrin, type XVIII collagen) (Fig. 9.2) and, lastly, serglycin, which is essentially present in intracellular secretory compartments (Sarrazin et al. 2011) but can be secreted into the ECM in the highly glycosylated form on appropriate stimuli (Korpetinou et al. 2014).

HSPGs have the ability to bind to a wide variety of bioactive molecules, such as growth factors, chemokines, cytokines, and angiogenic factors, with diverse affinities and specificities. Maintenance of a well-tuned balance of HSPGs is indispensable for normal functions, and disturbance of this balance leads to alterations of various biological processes in the cell microenvironment, such as ECM integrity and regulatory homeostasis (Fig. 9.3). One crucial role of heparan sulfate (HS) is to function as coreceptors for growth factors on the cell surface. The mechanism of HS-dependent signaling was first detected in FGF2 signaling and has been extensively studied since then. HS chains enhance the binding of the FGF ligand to its

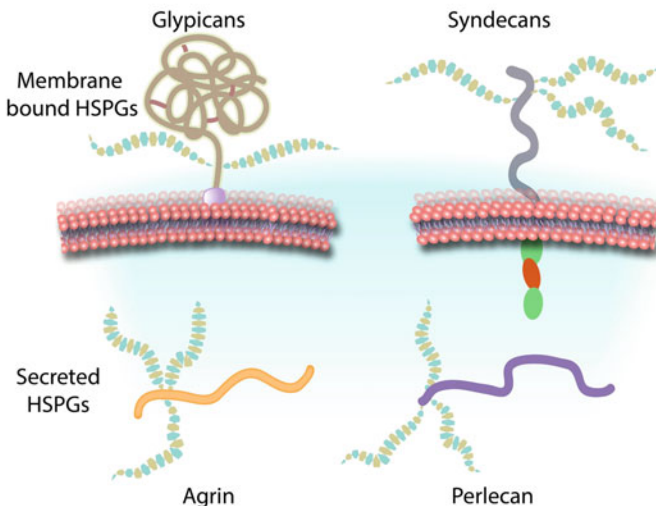

Fig. 9.2 Different types of heparan sulfate proteoglycans. Heparan sulfate proteoglycans (HSPGs) can be bound to the plasma membrane (glypicans and syndecans), or secreted (agrin and perlecan), or found in intracellular secretory compartments (serglycin, not shown in the figure)

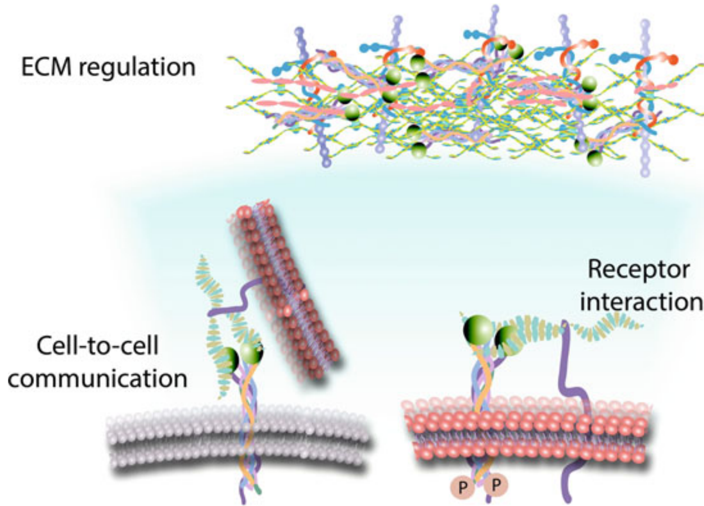

Fig. 9.3 HSPGs are involved in a multitude of biological processes to maintain homeostasis. HSPGs have the ability to bind to a wide variety of bioactive molecules and are involved in the regulation of the extracellular matrix (ECM) of the cellular microenvironment, in cell-to-cell communication, and in receptor interactions. The mechanism of HS-dependent signaling is for HS to function as coreceptors for growth factors on the cell surface, which is crucial for ligand-receptor binding kinetics for a variety of homeostatic processes

receptor (Rapraeger et al. 1991; Yayon et al. 1991) and is, therefore, crucial for ligand-receptor binding kinetics (Forsten-Williams et al. 2005). Similar signaling models were later described in many other pathways, including bone morphogenetic protein, WNT, Hedgehog, PDGF, and vascular endothelial growth factor (VEGF) signaling (Abramsson et al. 2007; Fager et al. 1992; Gengrinovitch et al. 1999; Molist et al. 1998; Reichsman et al. 1996; Ruppert et al. 1996; The et al. 1999).

HSPGs are composed of a core protein covalently O-linked with one or several HS GAG chains. Biosynthesis of HSPGs occurs in the Golgi compartment in several steps and involves multiple enzymes (Fig. 9.4). The process is initiated by the synthesis of a glucuronosyl-galactosyl-galactosyl-xylosyl linkage tetrasaccharide region. For this to form, first, xylose (Xyl) is transferred to specific serine residues of the core protein, e.g., syndecan or glypican, by xylosyltransferase-1/2 (Xylt1/2). Then, two galactose (Gal) residues are attached to the Xyl by galactosyltransferases-1/2 (GalT1/2). The linkage region becomes completed by attachment of glucuronic acid (GlcA) by glucuronyltransferase-1 (GlcAT-1). Up to this step, the tetrasaccharide is the same for all proteoglycans irrespective of whether they carry heparin/HS or chondroitin sulfate/dermatan sulfate GAG chains.

The first event in the HS chain elongation process is the attachment of an *N*-acetylgalactosamine (GlcNAc) residue to the tetrasaccharide linkage by exostosin-like-2/3 (EXTL-2/3). Then, a complex of EXT1 and EXT2 enzymes adds alternating GlcA and GlcNAc residues to the nascent chain (Busse et al. 2007).

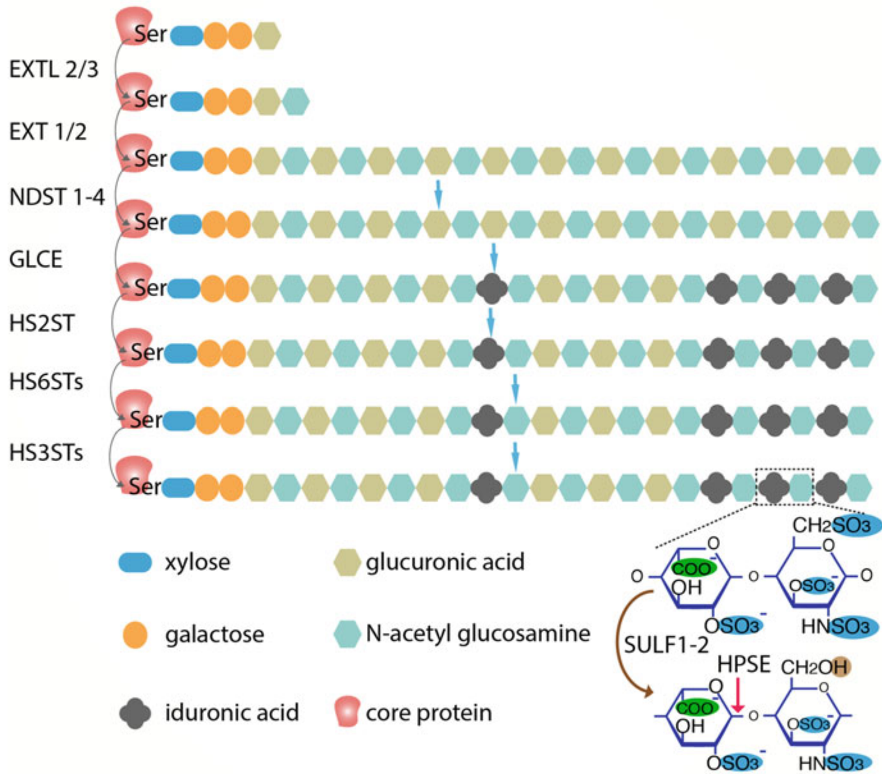

Fig. 9.4 Biosynthesis and modification of heparan sulfate (HS). Schematic representation of biosynthesis and modification of the nascent HS chain, showing the steps where the multiple enzymes are active. EXTL-2/3, exostosin-like-2/3; EXT 1–2, exostosin 1–2; NDST 1–4, *N*-deacetylase/*N*-sulfotransferase 1–4; GLCE, glucuronyl C5-epimerase; HS2ST, 2-*O*-sulfotransferase; HS6STs, 6-*O*-sulfotransferases; HS3STs, 3-*O*-sulfotransferases; SULF 1–2, sulfatase 1–2; HPSE, heparanase

As the HS chains are polymerized, their sulfation pattern is defined and shaped by a series of modification enzymes, which establish the distinct features by which the chains later interact with other molecules. The first modification is the *N*-deacetylation/*N*-sulfation of GlcNAc residues to GlcNS by *N*-deacetylase/*N*-sulfotransferases (NDSTs), which is also a prerequisite for most of the subsequent modifications (Kreuger and Kjellen 2012). In this step, the NDST enzymes remove *N*-acetyl groups from some of the GlcNAc residues and replace them with sulfate groups. This process generates a highly variable sulfation pattern across the HS chain, with some units remaining unmodified, while others become sulfated. Because several subsequent modifications occur at the N-sulfation sites, future ligand interactions of the HS chain depend heavily on actions by NDST enzymes at this stage.

Epimerization by glucuronyl C5-epimerase (GLCE) converts GlcA to IdoA in the N-sulfated regions of HS chains (Li et al. 2003). Thereafter, HS chains undergo O-sulfation at different sites by corresponding enzymes, 2-*O*-sulfotransferase (HS2ST), 6-*O*-sulfotransferases (HS6STs), and 3-*O*-sulfotransferases (HS3STs). The ability of HS to interact with proteins depends on the addition of these *O*-sulfate groups, and while there is only one 2-*O*-sulfotransferase, three 6-*O* sulfotransferase isoforms exist that have a slightly different preference for targets, but it is mainly the 6-OST1 that carries out the 6-O-sulfation of HS in vivo. There are seven 3-*O*-sulfotransferases, which are differentially expressed, both temporally and spatially (Cadwallader and Yost 2006), and can thereby influence mammalian development. After these modifications, the core protein with its attached HS is transported to the cell membrane and exported by exocytosis. Here, the modified domains constitute binding sites for various ligands. Once outside of the cell HS modification can further continue by removal of 6-*O*-sulfate groups by one of two endosulfatases (SULF1 and SULF2) (Ai et al. 2006). The SULFs lie in close association with the cell membrane, and through desulfation, these enzymes introduce postsynthetic modifications, which alter the biological function of HSPGs in normal homeostasis and oncogenesis. In particular, SULF2 has been associated with poor prognosis in lung carcinogenesis (Rosen and Lemjabbar-Alaoui 2010), and consequently, SULFs have been investigated as drug targets for pancreatic cancer (Alhasan et al. 2016).

The final step of HS postbiosynthetic modification is performed by heparanase (HPSE). HPSE, an endo-β-glucuronidase that cleaves the β-1,4-glycosidic bond between D-glucuronate and D-glucosamine, is the main degradation enzyme for HS. Its enzymatic action produces HS fragments (4–7 kDa) (Vlodavsky and Friedmann 2001) and, by that process, releases HS-bound ligands such as growth factors and cytokines, many of which play important roles in stem cells and CSCs. In normal homeostasis, extracellular HPSE levels remain low, and it is expressed only in platelets, mast cells, placental trophoblasts, keratinocytes, and leukocytes, but a marked increase in HPSE accompanies several diseases, such as inflammation and cancer (Vlodavsky et al. 2012).

The HPSE lifecycle is complex. Pre-HPSE is targeted to the lumen of the ER and then transferred as a 65 kDa pro-HPSE form that lacks catalytic activity in the Golgi. From here, vesicles bud off and carry HPSE to the cell surface. Once there, it interacts with HSPGs, is endocytosed in a complex with HS, and can be detected in late endosomes that fuse with lysosomes. It is in the lysosomes that HPSE is further processed and becomes activated by cathepsin L (Zetser et al. 2004). From here, active 50 kDa HPSE can be transported back to the Golgi to remodel HS, to the cell surface to degrade HS, or, alternatively, into the nucleus, where its function is not well understood. Nuclear translocation of HPSE has been reported during cell differentiation (Kobayashi et al. 2006) and in tumor cells (Nobuhisa et al. 2007). Pro-HPSE can also be bound in the glycocalyx of, for example, endothelial cells and play a role in glycocalyx remodeling in inflammation processes such as glycan shedding to facilitate adhesion of leukocytes (Lipowsky 2018).

9.5 Heparan Sulfate in Neural Stem Cell Commitment and Differentiation

In addition to regulating cell signaling, HSPGs have many other functions in cell homeostasis. They facilitate the transport of chemokines to present them on the cell surface. HS chains cooperate with integrins and adhesion receptors and are therefore integral in cell-ECM interaction and cell adhesion. Furthermore, HS chains are receptors for proteases and their inhibitors and regulate their activity and spatial distribution. Secreted HSPGs can even act as the backbone for stabilization of cytokines or as a shield from proteolytic cleavage in the ECM, thereby performing their role as signaling helper molecules (Sarrazin et al. 2011). Taken together, HSPGs are endowed with a capacity to integrate major processes in the body and thus have fundamental roles in normal stem cell maintenance and differentiation, both in healthy and pathological circumstances.

The neurogenic and tumorigenic niches are similar, and we previously reported that the composition of the ECM of the former undergoes changes during development (Bergstrom et al. 2014). HSPGs are the main components of the niche that modulate the activities of other factors, e.g., growth factors (reviewed in Xiong et al. 2014). We have also shown a vital role for HS biosynthesis in neural stem cell differentiation (Forsberg et al. 2012). A systematic overview of the requirements for glioma stem cell culture medium also points to the necessity for heparan sulfate, or heparin, as a stabilizer of FGF signaling (Zhang et al. 2020).

Using ES cell differentiation in vitro, the function of HSPGs in stem cell commitment and differentiation can readily be evaluated. Moreover, in normal ES cell differentiation, the regulation of N-, 3-O-, and 6-O-sulfation has been observed (Johnson et al. 2007). ES cells exhibit a low level of N-sulfation and increased expression of NDST4, HS3STs, and HS6STs during differentiation to NSPCs (Nairn et al. 2007). During differentiation from neuroepithelial precursors to neurons, the cells distinctly change their 6-O-sulfation pattern and HS chain length (Brickman et al. 1998). These discoveries suggest a role of sulfated HS in stem cell differentiation. When ES cells are differentiated using a monolayer differentiation protocol (Conti et al. 2005), they first undergo a differentiation into NSPCs, followed by the differentiation of NSPCs into mature neural lineages, i.e., neurons and glia. In our own studies, we observed that during the first differentiation phase, HPSE mRNA increases dramatically, followed by a gradual decrease during final differentiation. The latter coincides with a rise in the amount of HS during a phase when NSPCs are maturing into neurons and glia. Thus, the expression of HPSE is reduced, while the quantity of HS increases during neural differentiation (Xiong et al. 2017).

To understand the role of HS and HPSE in neural differentiation, ES cells with deletions or overexpression of biosynthetic enzymes and modifying genes have been used. The complete knockout of EXT1 causes the absence of HS chain synthesis, which has severe consequences for neural differentiation. EXT1-knockout ES cells have phenotypically normal colonies and a high expression of pluripotent markers, but depletion of EXT1 leads to differentiation arrest when subjected to monolayer

differentiation (Johnson et al. 2007; Kraushaar et al. 2010). Although EXT1-knockout ES cells can form embryonic bodies, they cannot generate terminally differentiated cells (Holley et al. 2011). When directing these ES cells to neural differentiation, the addition of soluble heparin can partially rescue differentiation to mature neurons (Johnson et al. 2007). In another ES cell line, using the knockdown of EXT1 with short hairpin RNA, soluble GAGs were found to be capable of inducing neural differentiation by influencing various RTK pathways (Pickford et al. 2011). NDST1/NDST2 double-knockout ES cells were reported to be completely devoid of N-sulfation but retained a very low level of 6-O-sulfation (Holmborn et al. 2004). Similar to EXT1-knockout ES cells, NDST1/NDST2 double-knockout ES cells maintain a normal phenotype and pluripotency in culture. However, they generally fail to differentiate upon embryoid body formation (Lanner et al. 2010). Angiogenic sprouting can occur in NDST1/NDST2-deficient embryoid bodies, but the adhesion of pericytes to nascent sprouts is reduced due to the dysregulation of TGFβ and PDGFB signaling (Le Jan et al. 2012).

When using stepwise protocols, by allowing the ES cells to first differentiate into multipotent progenitors, the NDST1/NDST2 ES cells surprisingly gave rise to osteoblasts, albeit with less efficiency than normal ES cells, and adipocytes could not be formed (Forsberg et al. 2012). Under conditions inducing neural differentiation, these ES cells appeared to be blocked at a primitive ectoderm-like state, expressing the early ectodermal marker FGF5 without proceeding to a neural progenitor lineage. The differentiation to neural precursors could be restored by a combination of heparin and FGF2 or FGF4, but this succeeded in a very narrow concentration range only (Forsberg et al. 2012).

When studying ES cells overexpressing HPSE, these cells show a faster proliferation potential, and they also formed larger teratomas in vivo than their wild-type counterparts. This increased growth rate was maintained during differentiation, as monitored by the monolayer protocol for neural induction, and they also showed enhanced activation of ERK and AKT pathways (Xiong et al. 2017). Interestingly, neural progenitors overexpressing HPSE were found to differentiate to a larger extent into oligodendrocytes than wild-type ES cells that hardly generated oligodendrocytes at all, and this increase was at the expense of neurons that were reduced, while the proportion of astrocytes did not change (Xiong et al. 2017). This shows that alterations in HS levels and composition can change how stem cells use various signaling pathways and consequently alter their differentiation potential.

9.6 Consequences for Brain Development When Heparan Sulfate Is Not Functional

It has been well studied that HSPGs are important in neural development, for example, in axon guidance. Mice with conditional EXT1 knockout in nestin-positive cells had serious errors in axon guidance of the major commissural tracts (Inatani

et al. 2003). When HS2ST or HS6ST1 were lost, it caused errors in the axonal navigation of retinal ganglion cells (Pratt et al. 2006) and critical malfunction of the corpus callosum with deregulated ERK signaling (Clegg et al. 2014). In addition, HS has also been shown to be a requirement for neural progenitor cell proliferation by modulating cell signaling. For instance, loss of HS2ST resulted in a reduced proliferation of precursors in the developing cerebral cortex (McLaughlin et al. 2003). When perlecan was ablated in the developing mouse brain it caused delayed progression of the cell cycle due to modified signaling by SHH and FGF2 signaling in neural progenitor cells (Giros et al. 2007). This also resulted in a reduction in the number of incorporated new neurons in the olfactory bulb (Kerever et al. 2014). Furthermore, syndecan-1 knockdown reduced neural proliferation by modulating the response to WNT ligands (Wang et al. 2012).

Mouse strains that overexpress HPSE or are devoid of HPSE, somewhat surprisingly do not exhibit severe phenotypes; they are fertile and have a normal life span, without prominent functional or pathological alterations. HPSE knockout mice were observed to exhibit an accumulation of long HS chains and showed a marked elevation in matrix metalloproteinase (MMP) (Zcharia et al. 2009). HPSE-overexpressing mice had a profound decrease in HS chain length and exhibited a reduction in food consumption and an accelerated hair growth rate. Additionally, they showed increased levels of urinary proteins, enhanced neovascularization, and disruption in epithelial basement membranes (Zcharia et al. 2004). There is very little information about HPSE in brain development, but Navarro et al (Navarro et al. 2008) reported HPSE levels to be high in the neocortex during early postnatal development. The same authors found that there is differential HPSE expression between different regions of the brain, and in the neocortex, the amount of enzymatically active HPSE was found to decrease sharply after the first 2 weeks after birth.

9.7 Glioblastoma

Malignant gliomas are the most frequent of primary brain tumors. They display an infiltrative growth pattern and respond poorly to radio/chemotherapy. The most common and also the most malignant glioma is grade IV glioma, also called glioblastoma (GBM). This highly aggressive tumor has only a medium survival time of 15-months for patients on the standard therapy, which consists of maximal safe resection, followed by radiation and chemotherapy (Stupp et al. 2005). Histologically, GBM is characterized by diffusely infiltrating tumor cells, a very high cellular density, microvascular proliferation, and the GBM hallmark; necrotic areas that are surrounded by pseudopalisading tumor cells. GBMs can be either primary or secondary. Primary GBMs account for 90%, in which case the tumor arises without a previous low-grade form, and secondary GBMs develop from a lower-grade glioma (Table 9.1).

Table 9.1 Key characteristics and classification of Glioblastoma

	Primary glioblastoma	Secondary glioblastoma
WHO Grade	IV	IV
Anatomical localization	Frontal (68%) and temporal lobe	Usually supratentorial, widespread distribution
IDH status	IDH wild type	IDH1/2 mutation
Median age of diagnosis	≈ 65	≈ 42
Histology	Loss of nuclear ATRX mutation rare, extensive necrosis	Extensive loss of nuclear ATRX mutation, limited necrosis
Genetic	CDKN2A, CDK4, CDK6, EGFR mutation, EGFRvIII rearrangement, H3F3A-G34 mutation, MET, MDM2 mutation, MDM4 amplification, NF1, PTEN, PIK3CA, PIK3R1 mutations, PTEN homozygous deletion, PDGFRA, TERT, TP53 mutations	TP53, TERT mutation, CDKN2A homozygous deletion
Chromosomal	Monosomy 10, trisomy 7 or gain in 7q	Trisomy 7 or gain in 7q, LOH 17p, 10q deletion
Epigenetic	G-CIMP, MGMT-promoter methylation	G-CIMP
Prognosis	Poor	Intermediate

GBM is characterized by extreme inter- and intratumoral heterogeneity (Chen and Hambardzumyan 2018). An important finding was the presence of mutations in the IDH (Han et al. 2020) (isocitrate dehydrogenase) gene of more than 10% GBM patients. Subsequent studies have now established IDH1 mutation as an independent factor for better prognosis and progression-free survival in GBM patients compared to their wild-type IDH1 counterpart. The Cancer Genome Atlas research network (TCGA) allows the molecular classification of adult GBMs according to gene expression patterns and mutational profiles of these tumors. This has led to the identification of distinct molecular subgroups of GBM (Table 9.2) (Verhaak et al. 2010; Wang et al. 2017). The proneural (PN) subtype harbors mutations in, e.g., PDGFRA, IDH1, and TP53. A signature feature of the PN subtype is also the expression of PDGFRA, NKX2-2, and OLIG2, which are all significant markers of oligodendrocyte differentiation. In a fraction of younger PN GBM patients, the tumors exhibit global hypermethylation (termed the glioma-cytosine-phosphate-guanine-CpG island methylator phenotype, G-CIMP) (Brennan et al. 2013), and they have a better prognosis than that associated with G-CIMP negative tumors. However, profiling the extent of DNA methylation across a larger patient cohort fails to show a consistent correlation between G-CIMP and IDH1 status (Brennan et al. 2013).

The classical subtype (CL) carries EGFR amplifications and homozygous deletion of Ink4a/ARF but generally lacks mutations in TP53. Furthermore, neural stem and progenitor cell markers are often expressed in CL GBMs. When CL subgroup GBMs are MGMT-methylated, they have a better response to temozolomide than non-MGMT-methylated cases (Noroxe et al. 2016).

Table 9.2 Molecular subgroups of glioblastoma (GBM)

Basis of classification	Subtype	Distinctive characteristics		
		Mutations	Chromosome	Gene expression
Molecular	Classical (CL)	*EGFR* amplifications, *Ink4a/ARF* homozygous deletion, loss of *CDKN2A*	Chr 7 amplifications, Chr 10 deletions	*MGMT* methylation EGFR, NES NOTCH
	Mesenchymal (MES)	*PTEN* loss, *NF1* homozygous deletion, *TP53* mutation, loss of *CDKN2A*	TERTp mutation Chr. 7 gain Chr. 10 loss	Mesenchymal markers (CHI3 L1/YKL40), astrocytic markers (CD44, MERTK), NF-κB target genes wound healing amd necrosis expression profile
	Proneural (PN)	*PDGFRA*, alterations, *IDH1* point mutations *TP53*	G-CIMP phenotype	Oligodendrocytic markers (PDGFRA, OLIG2, NKX2-2, SOX11, ASCL1)

Finally, the mesenchymal (MES) GBM subtype often has hemizygous deletions of NF1 and consequently, low NF1 expression. Typical inflammation-related markers (CHI3 L1/YKL40) and astrocytic biomarkers (CD44, MERTK) are highly expressed in MES GBMs. It has also been shown that EMT markers are often markers of the MES subtype (Zeisberg and Neilson 2009). In addition, MES GBM often shows high amounts of necrosis and gene expression profiles similar to wound healing and NF-κB target genes (Verhaak et al. 2010).

9.8 Medulloblastoma

Medulloblastoma (MB) is not very common, but are the most prevalent types of malignant pediatric brain cancers. They have been reported to arise from the undifferentiated neural stem or progenitor cells, mostly in the cerebellum (Gibson et al. 2010; Yang et al. 2008). The classification of MB is shown in Table 9.3. The histopathological classification of MB contains several groups. More than 70% of the cases are classical MB and characterized by dense small round cells with large nuclei and a small cytoplasm. Desmoplastic/nodular MB (D/N, making up 20% of patients was termed this way due to their high content of connective tissue in the internodular parts. Anaplastic/large cell MB (LC/A) is rarer, with 5% of the cases having a poorer prognosis than other MB types, and is defined by the presence of round cells with pronounced nucleoli, high numbers of mitotic figures, and high content of apoptotic bodies (Eberhart and Burger 2003; Holthouse et al. 2009).

Table 9.3 Classification of medulloblastoma (MB)

Basis of classification	Subtype	Prevalence (%)	Distinctive characteristics			Prognosis
			Major mutations	Chromosome aberration	Histological	
Histological	Classic	≈ 70			Dense small round cells with large nuclei and a small cytoplasm	Better
	Desmoplastic Nodular (D/N)	≈ 20			High content of connective tissue in the internodular regions	Better
	Large cell Anaplastic (LC/A)	≈ 10			Rounded cells with pronounced nucleoli, numerous mitotic figures	Poor
	MB with extensive nodularity (MBEN)	≈ 3–4			Expanded lobular architecture, low mitotic activity	Better
Molecular	WNT	≈ 10	*CTNNB1*, *DDX3X*, *SMARCA4*, *APC*	Loss of Chr 6	Classic	Better
	Sonic hedgehog (SHH)	≈ 30	*MYCN*, *GLI2*, *PTCH1*, *SUFU*, *MLL2*, *SMO*, *TP53*, *BCOR1*, *LDB1*, *GABRG1*	Loss in 9q, 10q, and, gain in chr 3q	Classic or D/N or LC/A	Intermediate
	Group 3	≈ 25	*MYC*, *PVT1*, *OTX2*, *MLL2*, *SMARCA4*, *CHD7*	Loss in Chr 5q, 10q, and chr 1q gain	Classic, LC/A	Poor
	Group 4	≈ 35	*OTX2*, *DDX31*, *CHD7*, *SNCAIP*, *MYCN*, *CDK6 GFI1/GFI1B*, *MLL2*, *KDM6A*, *MLL3*, *ZMYM3*	Loss of 17q chr X, 17p	Classic, LC/A	Intermediate

Finally, MB with extensive nodularity (MBEN) shows a better prognosis than other types (Polkinghorn and Tarbell 2007).

In addition to the histopathological classifications, as for GBM, distinct molecular subclasses of MB have been revealed. In contrast to GBM, however, where the molecular sub-classification has a weak relation to clinical outcome, in MB, the subclasses are increasingly used to guide treatment. This has led to more patient-specific treatments, and it has been possible to spare the children with the more benign MBs from the often harsh side effects that the extensive treatment causes to the young developing brain. Current knowledge divides MB into four subgroups; WNT (Wingless), SHH, Group 3, and Group 4. The best prognosis is that of the WNT group, and more than 90% of these patients have a longer survival period. WNT MB carries germline mutations of *APC*, a WNT pathway inhibitor, as well as somatic mutations in *CTNNB1* and high protein expression of β-catenin. The SHH group has a bimodal age distribution with an infant group and an adolescent group of patients. The SHH tumors have mutations in the SHH signaling pathways, including *PTCH*, *SMO*, and *SUFU*, as well as amplifications of *GL1* and *GL2*. Prognosis-wise, SHH MB has similarities to Group 4 MB and around 75% 5-year survival rate. Group 3 MB has the poorest clinical outcome and is an infancy tumor, while Group 4 MB occurs from infants to adolescents. These two groups have in common that >30% of the patients have a metastatic disease at diagnosis. The most prevalent genetic change in Group 3 MB is MYC amplifications, while Group 4 MB has MYCN and CDK6 amplifications (reviewed in Hovestadt et al. 2020). MBs have been further categorized into subtypes of already existing subgroups that reveal intertumoral heterogeneity through a combination of expression data and methylation analysis (Cavalli et al. 2017). This has made it possible to obtain a more detailed categorization of MB and has enabled risk stratification, which can give prognostic information. In addition, recent single-cell analysis of transcriptomes from MB of different molecular subtypes identifies different cells of origin for the different subgroups, which shows contribution of diverse cellular populations in the developing cerebellum to oncogenic conversion (Vladoiu et al. 2019).

9.9 Cancer Stem Cells Models of Malignant Brain Tumors

Because CSCs only make up a slight portion of the total tumor volume, whole tumors will not easily reveal information about CSCs, and therefore it is important to study and culture them separately. In addition, with the hierarchical structure proposed for CSCs, selection pressure and genomic instability will consequently lead to the development of new CSC clones formed over time, with different somatic variants. These dynamic processes lie behind a large part of the heterogeneity seen in, e.g., GBM, and the intratumoral heterogeneity underscores a necessity to have large numbers of CSC lines for studies and drug discovery. New drugs and/or drug combinations will be needed to target patient-specific CSCs in GBM, which to date remains an unmet medical need. This type of precision medicine was long

hampered, because valid cell models were not available (Lee et al. 2006), and screening for cancer therapeutics relied on traditionally serum-cultured brain cell lines that are not adequately mirroring the GBM patient's tumor. About a decade ago, it was confirmed that GBM cells, when grown in the same culture medium as neural stem cells, retain the characteristics of the original disease and can be expanded for large-scale studies (Pollard et al. 2009). We have been part of a collaborative effort in establishing the Human Glioma Cell Cultures (HGCC) resource, which consists of clinically annotated and experimentally validated CSC lines from GBM (Xie et al. 2015). In this biobank, we provide newly established, and well-characterized cell lines derived from GBM patient tumor tissue based on neural stem cell conditions. The cell lines constitute an open resource (http://www.hgcc.se) along with accompanying data for research and drug discovery.

As we have learned, serum-free culture conditions presumably conserve the characteristics of the original tumor, but it has been shown to be more difficult to culture MB compared to GBM using a neural stem cell medium. Therefore, many classical MB cell lines that were established decades ago are still used as in vitro models of the disease (reviewed in Ivanov et al. 2016). This also means that some of the MB subgroups are not fully represented when it comes to cell lines, such as the WNT MB and Group 4 MB that make up around 50% of the cases but are poorly represented as cell lines. However, a recent publication suggests that the serum-free culture of MB had a better success rate when cells were grown on high-adherence plastic in monolayers rather than as non-adherent sphere culture (Sanden et al. 2015). A more laborious method, but probably better reflecting the original tumor, is xenografting of MB cells, which often retain CSC properties (Dietl et al. 2016).

9.10 Heparan Sulfate in Brain Tumors and Brain Tumor Stem Cells

CSCs and neural stem cells have many traits in common, and early studies have provided compelling evidence that programs for stem cell differentiation rely heavily on a correctly sulfated HS. This can be exemplified by myoblast differentiation (Rapraeger et al. 1991) and is true also for stem cells of the hematopoietic lineages (Gupta et al. 1998). From genetically modified mice, we have learned that HS biosynthetic genes are vital for vertebrate development. For example, deletion of H2ST (Bullock et al. 1998) or NDST1 (Ringvall et al. 2000) are both lethal. In contrast, we are not aware of studies describing major stem cell disturbances in HPSE null mice, for which major phenotypical alterations are lacking. There is, however, increased branching of mammary glands in HSPE knockout mice and enhanced sprouting in an ex vivo neovascularization assay (Zcharia et al. 2009). In addition, HPSE knockout mice have alterations in macrophage activation due to less cytokine production, which causes reduced infiltration compared to wild type

(Gutter-Kapon et al. 2016). To what extent the HPSE deficiency as described above, affects stem cell maintenance and/or differentiation is not known.

HS is critical for the transition of self-renewing ES cells to progeny that initiates differentiation to committed cell fates (Kraushaar et al. 2010). This is illustrated by the ability to form the neural lineage (Johnson et al. 2007) or in vascular development (Jakobsson et al. 2006). It is not only the presence of HS as such which is important, but it is the sulfation pattern, which becomes more elaborate with increasing sulfation when ES cell differentiate (Pickford et al. 2011). Therefore, it is proposed that CSCs, being similar to neural stem cells, carry HS with a lower degree of sulfation than do mature neural cells and that this provides survival advantages to CSCs.

High HPSE expression in GBM CSCs (Kundu et al. 2016) or breast cancer CSCs (Ibrahim et al. 2017) confirm this hypothesis. HPSE is highly expressed in GBM stem cells, and both the 50-kDa form, which carries the enzymatic activity and its precursor, the 65-kDa, latent form, were detected (Kundu et al. 2016). When we downregulated HPSE, either using shRNA or the compound PG545, which is an HPSE inhibitor, it resulted in reduced cell numbers, showing HPSE to be important in GBM cells. When the different subtypes of GBM, both within our own GBM cell bank and in the TCGA dataset of GBM tissues, were queried for HPSE expression, we found MES GBM to have the highest levels, while CL GBM was the lowest with regard to HPSE mRNA (Kundu et al. 2016). It can therefore be suggested that different HPSE levels are part of the GBM heterogeneity. In addition, others have reported that tolerance to stress and resistance to chemotherapy, well-known features of CSCs, result from overexpression of HPSE in GBM cells (Shteingauz et al. 2015).

So far, there is no extensive multi-omics analysis available of proteoglycans in brain tumors, but the TCGA has been queried for mRNA expression data in its GBM cohort, for both the proteoglycan core proteins and the biosynthetic as well as modifying enzymes for HS and CS (Wade et al. 2013). In their paper, many differentially expressed genes were presented that can potentially map proteoglycan expression in tumors versus non-tumor tissue and between molecular subclasses of GBM. In addition to mRNA profiles, mass spectrometry and Raman microspectroscopy have recently been employed for the purpose of brain tumor characterization. Tran et al. (2017) have shown that the content and structure of HS disaccharides differ widely between GBM spheres cultured from different patients, using liquid chromatography-mass spectrometry. It is a promising study, and it could be expanded to systematically measure GBM inter-and intra-patient heterogeneity, with regard to proteoglycan expression, to investigate which GBMs would be more responsive to HS modifications. In a recent paper, Kopec et al. (2019) measured the glycocalyx in tumor tissue from an MB patient with Raman spectroscopy and imaging. Proteoglycans could thus be identified by virtue of their unique vibrational signatures. This expands the methods for analyzing HS in brain tumors.

Steck et al. were among the first to describe a role for HS in glioma (Steck et al. 1989), where they found higher production of HS in high-grade glioma cells than in control, non-tumor cells, and also that GAGs were released into the culture medium. In the same study, they described intense and diffuse staining of HS, localized to the

surface of the cell, in contrast to the case of normal cells or low-grade astrocytoma, which displayed punctate HS staining. In an investigation of glioma specimen, high GAG levels in particular HS and DS were found to be more abundant, compared to the non-tumor brain (Bertolotto et al. 1986).

The role for chondroitin sulfate proteoglycans (CSPGs) must also be mentioned since NG2, one of the first identified and most commonly used markers for oligodendrocyte precursor cells, is also known as CSPG4 (Nishiyama et al. 1999). In an early study, NG2/CSPG4 was shown to be overexpressed in glioma (Shoshan et al. 1999). Oligodendrocyte precursors are one of the cells of origin for glioma (Lindberg et al. 2009), and due to the overexpression of NG2 in GBM, it has even been proposed as a prognostic marker (Svendsen et al. 2011). Versican, another CSPG, was found to be highly expressed in GBM (Wade et al. 2013). This was later corroborated in both mouse and human glioma (Hu et al. 2015). In fact, versican, along with other ECM genes, was classified as a signature gene to predict invasiveness in low-grade glioma (Sainio and Jarvelainen 2014). One of the versican-mediated functions in glioma is migration, as anti-versican antibodies reversed migration stimulated by TGFβ2 (Arslan et al. 2007).

Because hyaluronan is a major component of the brain ECM and is commonly linked to inflammation and cancer, it is not surprising that its main receptor, CD44 (Aruffo et al. 1990), is highly expressed in the TCGA cohort of GBMs (Wade et al. 2013). Furthermore, CD44 is a marker especially for the MES subtype (Phillips et al. 2006) and has even been shown to induce neuronal degeneration when secreted from GBM (Lim et al. 2018). CD44 is also widely used for identifying and isolating CSCs in GBM (Anido et al. 2010), and has been demonstrated to regulate critical stemness parameters. Moreover, since serglycin has been identified as a ligand for CD44 several studies have implicated the serglycin/CD44 axis to have an important role in tumorigenesis, including stem cell self-renewal and ECM remodeling (Chu et al. 2016). Serglycin expression can also support cancer stem cells, by induction of NANOG, in lung cancer (Guo et al. 2017), and it is interesting that deregulation of this normally hematopoietic granule HS can be a prognostic marker in NSCLCs. Serglycin has also been demonstrated to be expressed in high levels in high-grade glioma compared to low-grade gliomas (Roy et al. 2017).

Phosphacan, or protein tyrosine phosphatase receptor β/ζ (PTPRZ1), is another highly expressed gene in the TCGA GBM cohort. Several studies have shown that this receptor is involved in glioma, for example, a fusion transcript between PTPRZ1 and MET was detected (Bao et al. 2014) and shown to increase resistance to radiation and chemotherapy (Zeng et al. 2017). Furthermore, glioma formation in mice can be inhibited by a small molecule inhibitor of PTPRZ1 (Fujikawa et al. 2016), making it an interesting PG to study in GBM.

Syndecans are integral membrane proteins with intracellular signaling modules, while glypicans are tethered to the membrane by a GPI anchor and lack intracellular contact. These two families of HSPG core proteins are altered in glioma as shown by Su et al. (2006) for glypican-1 and for syndecan-1 by Watanabe et al. (2006). The latter found that syndecan 2-4 have a ubiquitous expression in the normal brain and that it does not change in glioma, while syndecan-1 is only expressed in glioma and

not in non-tumor brain (Watanabe et al. 2006). High syndecan-1 has furthermore been correlated to shorter survival times in glioma, and among the 100 patients in the study, the authors found grade IV gliomas to have the highest expression (Xu et al. 2012).

Several members of the family of small leucine-rich proteoglycans (SLRP) are highly expressed in GBM, such as fibromodulin, biglycan, and lumican. Decorin, on the other hand, has very low expression. This SLRP is present in the ECM, where it interacts with a variety of membrane receptors and cytokines. The role of decorin in glioma is not fully elucidated but mostly it has been reported to suppress glioma. For example, decorin inhibited adhesion and migration and induced autophagy in glioma cells in vitro (Yao et al. 2016). Culturing GBM cells as spheres in neural stem cell medium induced expression of both decorin and lumican, and interestingly, these CSC-like GBM cells with high SLRP expression developed temozolomide resistance, while the parental cell line was sensitive to chemotherapy (Farace et al. 2015). Fibromodulin, was identified as a regulator of glioma in a screen for epigenetically controlled genes in GBM (Mondal et al. 2017). The authors found an effect on glioma migration by secreted fibromodulin and that this effect was downstream of TGFβ1.

It remains to be experimentally validated how HS biosynthetic enzymes influence GBM and GBM stem cells. It should be noted that the expression of *NDST* 1–4 is very low in GBM, which would suggest less sulfation in GBM cells than in normal cells of the mature brain (Wade et al. 2013). In fact, of the 15 biosynthetic enzyme genes for which data is available in the TCGA, the vast majority is downregulated (Wade et al. 2013). As for the 3-*O*-sulfotransferases, two out of four are upregulated, while two are downregulated. In this enzyme family, *HS3ST3a1* is the most upregulated of all HS biosynthetic genes in. A previous study had found it to be high in glioma cells but not in normal human astrocytes (Su et al. 2006). All the 6-*O*-sulfotransferases were downregulated (Wade et al. 2013), and Ushakov et al. (Ushakov et al. 2017) also report lower mRNA levels of HS6ST1 and HS6ST2 in both grade III and grade IV glioma when they compared to non-tumor brain tissue from the same patients. The clinical consequences of the above differences in sulfation pattern remain to be clarified.

Once the HS chain is synthesized and modified, it is further edited by the sulfotransferases SULF-1 and SULF-2. Both of those are upregulated in GBM (Wade et al. 2013). The function of SULF2 is to remove 6-*O*-sulfate moieties, and this step activates signaling pathways. One of the common denominators of GBM is aberrant activation of receptor tyrosine kinases. Because HS-GAGs are negatively charged, they possess the ability to bind to a multitude of growth factors. PDGFRA activation is a typical example herein, because it is frequently amplified in GBM, and both PDGFA and PDGFB are often overexpressed in glioma. In fact, PDGF is one of the known driver genes in GBM, and several studies have shown this, e.g., using genetically modified mice (reviewed in Shih and Holland 2006). One of the best examples of the role of HS in GBM is the *SULF2* gene (Phillips et al. 2012). In this study, the authors find SULF2 to be highly overexpressed in tumor tissue from GBM patients and in patient-derived cell cultures. They also show that suppression of

SULF2 expression results in smaller GBM xenografts in mice. This effect was most pronounced in PN GBM, which are often dependent on PDGFRA signaling but not in CL GBM that is to a large extent driven by EGFR activation. SULF2 was one of the genes identified in a retrovirus-driven PDGF mutational insertion that caused glioma in mice (Johansson et al. 2004), and hence, the SULF2 overexpression identified by Phillips promotes HSPG-dependent PDGFRA signaling.

It is interesting to note that many proteoglycan genes that have already been shown to support cancer progression, for example, in the metastatic process, were found to be highly regulated upon examination of the TCGA cohort (Wade et al. 2013). Among those, both membrane-bound proteoglycans and secreted ones are found. Because many, but not all, proteoglycans are expressed at a higher level in GBM than in normal brain tissue, it suggests that proteoglycans and their synthesizing and degradation enzymes could be biomarkers for GBM.

As mentioned earlier, HPSE is the enzyme that degrades HS, thereby releasing molecules that are bound to the HS that can then act in the microenvironment to promote cancer growth and spread. HPSE has been identified as a major molecule in invasion and metastasis of a wide range of tumors (Vlodavsky et al. 2012), and we and others have found HPSE to be overexpressed in glioma (Hong et al. 2010; Kundu et al. 2016) and in MB (Sinnappah-Kang et al. 2005; Spyrou et al. 2017). Our recent publication shows that HPSE stimulates several signaling pathways through interaction with the glycophosphatidylinositol-linked sialoprotein CD24 (Barash et al. 2019).

Several groups have studied HPSE expression and function in GBM, although the results have been somewhat contradictory. mRNA expression of HPSE was found to be high in oligodendroglioma, anaplastic astrocytoma, and GBM, compared to non-tumor brain (Hong et al. 2010), but no clear correlation to WHO malignancy grade was observed. Similarly, we detected high HPSE protein levels in a cohort of >180 glioma patients whose tumors were of different WHO-grades. Interestingly, in our study, high-grade gliomas were more strongly stained in the neuropil than low-grade gliomas (Kundu et al. 2016). Another study, in contrast, finds no HPSE in human GBM tissue, and furthermore, when the authors xenografted the GBM cell line U87MG, that expresses HPSE in vitro, to the mouse brain for tumor formation, tumors developed, but the cells had lost their HPSE expression (Ueno et al. 2005). However, the use of different HPSE antibodies for immunohistochemistry could perhaps explain the contradictory results.

In yet another study using the U87 GBM, the investigators demonstrated that a modest increase in HPSE made tumors grow larger after xenografting (Zetser et al. 2003). When HPSE was overexpressed in the U251 GBM cell line, it resulted in the increased invasion, colony formation, and phosphorylation of AKT (Hong et al. 2008). We have observed that when HPSE was downregulated in patient-derived GBM cells grown under neural stem cell conditions, it reduced proliferation in vitro, while exogenous addition of HPSE instead supported cell growth and the activation of intracellular signaling pathways such as ERK and AKT (Kundu et al. 2016). It is possible that the use of different cell lines, i.e., traditionally serum-cultured lines

(e.g., U87) versus newer, patient-derived stem cell cultured GBM cells (http://www.hgcc.se) could explain some of the different results.

We furthermore induced orthotopic glioma in HPSE transgenic and HPSE knockout mice and found that the levels of HPSE in the mouse brain determined the size of the resulting glioma. Therefore, we propose that HPSE, either associated with the cell surface or secreted HPSE promotes glioma invasive properties and therefore enhances tumor progression when HPSE is present in the tumor microenvironment. Similar results to those in GBM were obtained when studying HPSE in medulloblastoma and other embryonal tumors. These pediatric brain tumors had very high levels of HPSE protein, while the normal brain stained weakly for HPSE. MB cell lines treated with HPSE grew faster, and HSPE inhibition resulted in inhibition of tumor cell migration and invasion (Spyrou et al. 2017). In both GBM and MB, we found that HPSE in its latent, 65 kDa form, after being intracellularly processed into the 50 kDa form, to become enzymatically active, was able to rapidly stimulate downstream signaling pathways (Kundu et al. 2016; Spyrou et al. 2017). This suggests that HPSE in brain tumors has both enzymatic and non-enzymatic effects.

To better understand the mechanisms behind the stimulatory effects by HPSE, we used an in vitro model with inducible HPSE overexpression in U87 GBM cells. By differential gene expression analysis, we found the glycophosphatidylinositol-linked sialoprotein CD24, a cell adhesion molecule to be highly upregulated by HPSE (Barash et al. 2019). From staining of our GBM cohort, we identified patients with high HPSE and high CD24 and found that their survival time was lower than for patients with high HPSE and low CD24. Furthermore, when overexpressing CD24, the glioma cells grew more aggressively and formed larger tumors

as xenotransplants. Distribution of antibodies to CD24 or HPSE reduced tumor size. A ligand to CD24, L1CAM, was identified as a member of the complex formed, and anti-L1CAM antibodies could attenuate tumor growth in vivo (Barash et al. 2019). Thus, an HPSE-CD24-L1CAM axis exists in glioma, which could be a potential drug target.

There are already several efforts worldwide to target HPSE. One inhibitor of this enzyme is PG545 (Dredge et al. 2011), which we used to efficiently block tumor formation in vivo concomitantly with reduced tumor cell proliferation, induction of apoptosis, and reduced tumor angiogenesis (Spyrou et al. 2017). Because normal human astrocytes were not affected by the HPSE treatment, and because only cancer cells have high HPSE levels, the specificity of this molecule is promising. In addition, several studies have shown that HPSE treatment of malignant tumors of various kinds are sensitive to HPSE inhibition, both due to the effects on the tumor cells themselves and the effect in the tumor microenvironment. However, for brain tumors, PG545 will likely not be a candidate drug because of its inability to cross the blood brain barrier (Spyrou et al. 2017). However, it is clear that HPSE plays important roles in the progression of brain tumors and that this enzyme can constitute a therapeutic target using other approaches.

9.11 Concluding Remarks

This chapter summarizes the current knowledge about the role of HS in neural stem cells and brain tumor stem cells. We describe how neural stem cells are regulated and how the concept of cancer stem cells applies to brain tumor biology. Our emphasis is on the specific ECM of the brain, where HS is an integral molecule for a multitude of signaling pathways involved in both normal stem cells and brain tumor stem cells. Furthermore, we review the biosynthesis, modification, and degradation of HS and how the enzymes involved in these processes, as well as the HSPG core proteins, are co-opted by brain tumors to promote malignancy and formation of the tumor microenvironment. Reviewing the current literature, we suggest that HSPGs are important biomarkers of brain tumors and that they constitute novel potential therapeutic targets. We believe that pharmacological blocking of cancer-induced changes in HSPG has the potential to inhibit both oncogenic signalings in tumor cells and to disrupt the interaction between brain tumor cells and their highly specialized microenvironment, which is critical for rapid growth and invasiveness of these malignant tumors.

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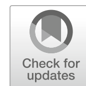
Chapter 10
The Hyaluronic Acid–CD44 Interaction in the Physio- and Pathological Stem Cell Niche



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Abstract The CD44 protein is a single-chain transmembrane glycoprotein able to bind several ligands. Hyaluronic acid is its main ligand and this interaction is the most extensively studied. Hyaluronic acid, as a component of the extracellular matrix, through CD44 is able to modulate several mechanisms that control stem cell behavior, such as migration and anchorage, efflux of toxic compounds, growth in hypoxic conditions and quiescence, besides their self-renewal and differentiation potential. In this chapter we describe the hyaluronic acid–CD44 interaction as part of the communication between the extracellular matrix and different cells that belong to the stem niche, focusing on the hematopoietic and adipose niche. Finally, we discuss the role of these interactions in the transformation of the normal stem niche to a tumorigenic niche that allows the development of cancer stem cells.

10.1 Introduction

The stem cell niche is a specialized physical localization within the tissues where stem cells are residing. Stem cells are in contact with different types of cells and components of the extracellular matrix (ECM). The composition of ECM provides several extrinsic mechanisms that strongly control stem cell behavior. The major

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ECM components include proteins, collagens, glycoproteins, and proteoglycans and glycosaminoglycans, like hyaluronic acid (HA) and heparan sulfate, respectively. These components are able to interact with stem cells by direct binding to different receptors or indirectly through noncanonical growth factor presentation (Pardo-Saganta et al. 2019). CD44 is a key biomarker in stem cells; it is an adhesion molecule that plays an important function in driving their behavior. The CD44 protein is a single-chain transmembrane glycoprotein able to bind several ligands, such as extracellular components (fibronectin, laminin, osteopontin, HA), cytokines and growth factors. The biological and molecular function of CD44 in maintaining the stem cell state is associated with mechanisms of cell adhesion, anchorage, and homing by induction of different intracellular signals. The association with these mechanisms occurs both under physiological and pathological conditions (Morath et al. 2016). In the latter, CD44 could act by sensing the ECM changes or alteration of the niche, as well as induce signals that conduct toward a variation in the state of cell stem. In this chapter, we describe several aspects of the HA–CD44 interaction in its role of maintaining the stem cell phenotypes as part of the communication between the ECM and different cells that belong to the stem cell niche. Besides, we discuss the role of this interaction that facilities the transformation of a healthy stem cell niche to a tumorigenic niche (Fig. 10.1).

10.2 The Stem Cell Niche: Types of Niche and ECM Aspects

The stem cell niche could be in general described as an anatomical and dynamic structure composed of the stem cell itself, stromal cells, soluble factors, neural inputs, vascular network, and ECM components, which are interplaying to maintain the stem cell properties (Jones and Wagers 2008).

The ECM is a crucial player in the niche whose composition affects stem cell differentiation by several mechanisms (Gattazzo et al. 2014). Physically, the ECM offers support for the cells within the basement membrane, mainly type IV collagen, laminins, fibronectin, and the interstitial zone composed of fibrillar collagens, glycoproteins, proteoglycans, and glycosaminoglycan as HA. Both regions are differentiated according to the tissue, but in general, they have different physical properties referring to rigidity, porosity, solubility, stiffness, and topography (spatial arrangement and orientation) that impact on the mechanisms associated with cell division, tissue polarity, and migration (Watt and Huck 2013). Besides, the ECM components protect the stem cells for maintaining their DNA integrity, an adequate O_2 tension, modulate gradients and accessibility to growth factors, and thus to control autocrine and paracrine signals for stem cells homeostasis and to avoid cell transformation (Pardo-Saganta et al. 2019). Moreover, the ECM composition affects its biochemical properties, referred to its capabilities to induce intracellular signals, regulating changes in gene expression that, in turn, control the cell behavior.

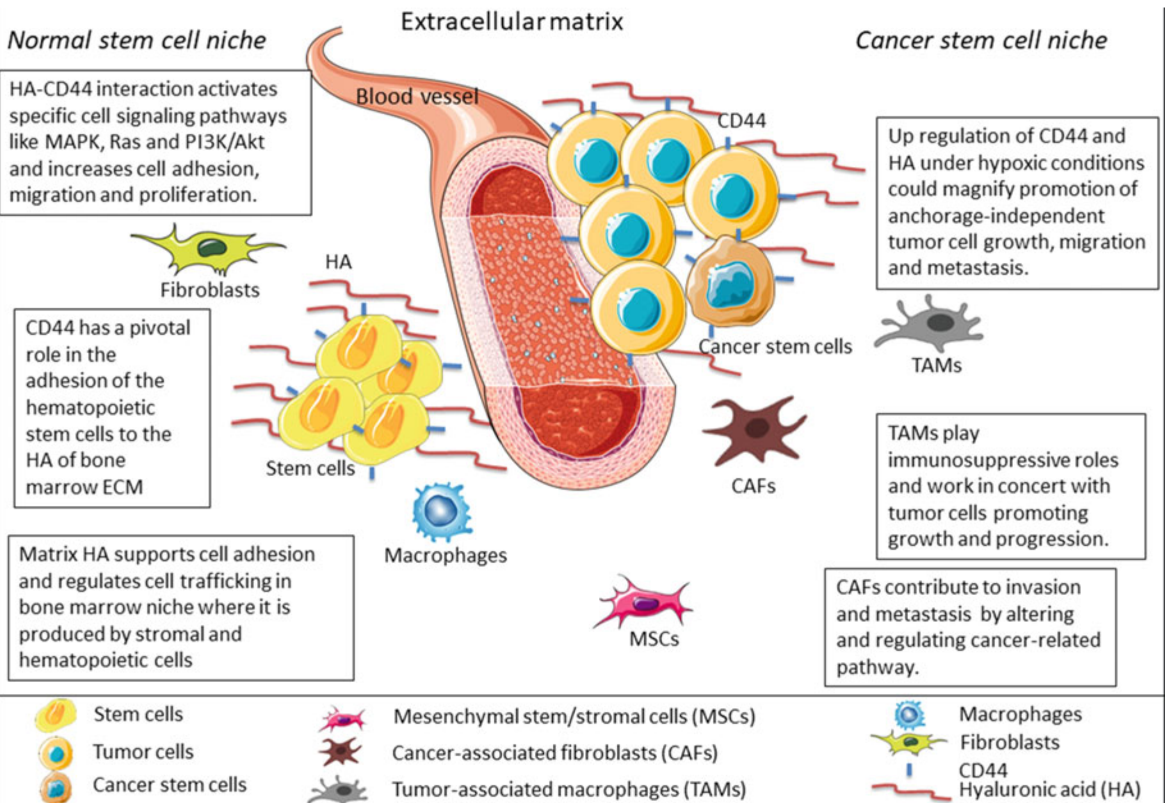

Fig. 10.1 The stem cell niche. The stem cell niche is a specialized region within the tissue that represents a dynamic structure composed of the stem cell and other non-stem cells (like stromal and immune cells), soluble factors, vascular network, and extracellular matrix components (like HA). CD44 can be found on the surface of stem cells and cancer stem cells. HA is the most specific ligand for CD44 activation, which can lead to cell–cell and cell–ECM interactions associated with different physiological and pathophysiological processes. MSCs are present in a variety of tissues and can be found in stem cell niches as well as

10.2.1 Different Stem Cell Niches

In recent years, many different stem cell niches have been identified, including the hematopoietic, epidermal, intestinal, muscular, adipose, and neural stem cell niches. Even though every stem cell niche has its characteristics necessary for maintaining the stemness of those particular cells, there are underlying mechanisms as well as cellular and acellular components that are typical for all stem cell niches (Gattazzo et al. 2014). Here we describe these components in the hematopoietic niche as one of the most described niches and adipose niches that represent one of the latest findings.

Long bones, like the femur, contain the bone marrow (BM), which is where hematopoiesis takes place and bone formation occurs. In this context, several stromal cell populations compose the BM tissue and participate not only in hematopoietic regulation but are also major actors in maintaining bone homeostasis (Bolontrade and García 2016). Initial investigations with MSCs precursors led to the paradigm of a BM niche that hosts hematopoietic stem cells (HSCs) and supports hematopoiesis. Within this niche, two different kinds of stem cells, HSCs and MSCs, share standard features such as a hierarchical organization (Owen 1978). Both stem cells interact with each other modeling the BM niche and generating the inductive stimulus that permits hematopoiesis. Given the heterogeneity of the niche in terms of cell composition, "sub-niches" or sublocations could be determined. Consequently, some MSCs are in close communication with HSC and other MSCs subpopulations and are functionally and anatomically related to bone generation (Mendez-Ferrer et al. 2010). This contact between HSCs and stromal cells, such as MSCs could be a clue for stemness maintenance. Actually, both types of cells produce HA, which is the major glycosaminoglycan component of the BM matrix. HA plays a key role in providing structural support. Different receptors can bind HA in this environment. However, the most recognized is CD44-hematopoietic (CD44H), which has a high but not constitutive affinity for binding HA (Haylock and Nilsson 2006). The CD44–HA interaction promotes adhesion, homing, and migration of HSC as well as HSC quiescence and resistance to low oxygen pressure. It has also been shown that the apoptosis resistance of leukemia-initiating cells (LIC) presents dependence on the CD44-HA crosstalk (Nilsson et al. 2003; Zoller 2015). In the specific niche of the BM, CD44 is a major regulator of chemotaxis. CD44 as previously stated is involved in cell adhesion and has been reported to play significant roles in the hematopoietic regulation by the participation of CD44-HA in the anchorage of HSCs to the endosteal region. This process is highlighted by the fact that CD44 could be regulating not only HSC homing but also contributing in this way to the migration of these stem cells to the specific niche that might, in turn, regulate their fate (Cao et al. 2016).

Fig. 10.1 (continued) cancer stem cell niches. MSCs can be integrated into these niches after recruitment and interact with different cells. HA, hyaluronic acid; ECM, extracellular matrix; MSCs, mesenchymal stem/stromal cells

Research focus in the BM niche resulted in the paradigm concept that states that a given stem cell will communicate with the surrounding niche, exchanging specific chemical signals with that niche (tissue) and thus determining the stem cell fate, i.e., generating a differentiated progeny educated by that tissue. Crosstalk signals are then controlling and determining a spatio-temporal environment, the stem cell niche, and this feature is shared by all tissues where cells with stem capacity reside (Spradling et al. 2001).

MSCs are residents of a variety of tissues (Meirelles et al. 2006). At the beginning of this century, the concept of adipose tissue residing MSCs appeared (Zuk et al. 2001). Thus, adipose tissue was first described as mainly associated with fat reservoir functions (Luo and Liu 2016). Nowadays it is widely accepted that adipose tissue is not just an organ to store energy, but it is also involved in the maintenance of systemic metabolic homeostasis. It has also been implicated in different pathological contexts through the release of specific molecules, adipokines, which are overexpressed in this tissue (Bulcão et al. 2006). The predominant form of fat in adults is represented by white adipose tissue and it comprises two main components: a stromal vascular fraction, composed of different stromal cellular populations such as adipose tissue MSCs (AT-MSCs), fibroblasts, preadipocytes, endothelial cells, pericytes and immune cells, and the second component represented by grouped spherical adipocytes (Torres et al. 2016). These two fractions cooperate to maintain the integrity of the tissue by allowing the crosstalk between different cell populations within the adipose stroma. In order to maintain tissue homeostasis essential biological processes are involved, such as adipogenesis. In white adipose tissue, this process requires the differentiation and proliferation of MSCs within the adipose niche. These MSCs are localized near blood vessels or capillaries in the perivascular niche, adopting a pericyte-like phenotype but still expressing stem cell markers (Lin et al. 2008). As mentioned above, stem cells integrate specific chemical signals from the surrounding niche or tissue and respond by generating a cell progeny educated by that tissue (Pope et al. 2016). Between the signals that trigger MSCs differentiation toward the adipoblastic lineage, two of them stand out because of their relevance: The WNT and the hedgehog pathways (Lowe et al. 2011). Actually, CD44 is one of the gene targets of Wnt activation, which in turn acts as a regulator of the Wnt-induced/β-catenin signaling pathway (Schmitt et al. 2015). During the differentiation process, adipose stem cells not only need to differentiate but also need to leave the perivascular niche and get in contact with the adipose-associated ECM. (Liu et al. 2005; Noro et al. 2013).CD44 is associated with the adipose tissue stem niche, being a surface marker of AT-MSCs (Mildmay-White and Khan 2017). Another critical and necessary cue for the differentiation of preadipocytes is lipid accumulation, which is an insulin-dependent mechanism, in which endothelial cells induce the secretion of lipoprotein lipase by mature adipocytes, resulting in the liberation of lipids from lipoproteins in adipose tissue context (Knutson 2000). HA has been reported to influence adipogenic differentiation, given that supplementation of HA in culture medium favored the differentiation of preadipocytes to mature adipocytes. On the other hand, the reduction of HA in murine preadipocytes by the

use of hyaluronidases or pharmacological inhibitors resulted in the inhibition of adipose differentiation (Wang et al. 2014; Zhu et al. 2019).

All these points out the complexity of the adipose stem cell niche and how some of the signals necessary for differentiation induction are associated with the environment or niche, where CD44 plays an essential role in maintaining stem cell phenotype, by allowing the interaction between stem cells and the ECM components.

10.3 CD44: The Stem Cell Marker and the Main Hyaluronan Receptor

CD44 is a family of non-kinase, single-span transmembrane glycoproteins widely expressed on both embryonic stem cells as well as mature cells, such as leukocytes, fibroblasts, epithelial cells, keratinocytes, and endothelial cells (Gronthos et al. 2001; Domev et al. 2012). Actually, CD44 expression is also upregulated in specific subpopulations of cancer cells and is recognized as a molecular marker for stem cells and cancer stem cells (CSC) (Yin et al. 2016). CD44 has the ability to bind numerous ligands including hyaluronan (HA), osteopontin, chondroitin sulfate, fibronectin, and collagen, among others (Weber et al. 1996; Faassen et al. 1992; Jalkanen et al. 1992; Toyama-Sorimachi et al. 1995). Nevertheless, HA is the most specific ligand for CD44 activation, which leads both cell–cell and cell–ECM interactions implicated in a wide variety of physiological and pathophysiological processes.

10.3.1 Structure: Exon Composition and Functional Domains

CD44 receptor is encoded by a single and highly conserved gene located on chromosome 11 in humans and on chromosome 2 in mice (Screaton et al. 1992). The human CD44 gene is located on the short arm of chromosome 11 and it is comprised of 19 different exons, while the murine CD44 gene has 20 exons since a homolog of exon six or variant one is not found in humans (Naor et al. 2002). Once synthesized, CD44 peptide has extracellular and transmembrane domains in common with a membrane-proximal region and a cytoplasmic tail (Idzerda et al. 1989). These regions have particular binding and regulatory sites tightly associated with CD44 function, and interestingly, different CD44 domains can be subject to different variations responsible for the high heterogeneity observed in this family of receptors.

The variability of the protein products obtained from the CD44 gene is due to both alternative splicing and posttranslational modifications. Extensive alternative splicing generates multiple isoforms of CD44, which affects mainly the extracellular

and membrane-proximal regions of this receptor. The first five and the last five exons of the human CD44 gene are non-variable and encode the shortest isoform of CD44: the CD44 standard (CD44s). The middle nine exons undergo the alternative splicing (as a single variant exon or combined with other exons) and assembled with the ten exons contained in the standard isoform, generating the CD44 variant isoforms (CD44v) (Ponta et al. 2003). Alternative splicing occurs only in particular cell types and activation states. Even more, it has been demonstrated that the inclusion of variant exons is dependent, in part, on mitogenic signals that specifically regulate alternative splicing (Li et al. 2014; Matzke-Ogi et al. 2016).

The encoded CD44s peptide contains an extracellular domain comprised of 248 amino acids including the amino-terminal region. This domain is moderately conserved among mammalian species and is implicated in CD44 function since it contains the HA binding motif. As a consequence, all isoforms of CD44 (both CD44s and CD44v) are capable of binding HA efficiently. Furthermore, all variants of CD44 share a highly conserved transmembrane domain and the cytoplasmic tail (Isacke and Yarwood 2002), which present motifs that are crucial for CD44 subcellular localization and the interaction with the cytoskeleton by ERM proteins (ankyrin and ezrin, radixin, moesin) (Naor et al. 2002). However, the membrane-proximal region is relatively non-conserved. As stated above, the heterogeneity of the CD44 receptors is also due to different posttranslational modifications, which are also specific, according to cell type and growth stage. The extracellular domain of CD44 includes several sites for *N*- and *O*-linked glycosylation as well as the addition of GAGs such as heparan sulfate or chondroitin sulfate (Bennett et al. 1995; Greenfield et al. 1999). For example, human hematopoietic stem/progenitor cells (HSPCs) express a glycoform of CD44: HCELL, providing the potent capacity to bind E- and L-selectin (Sackstein 2011). Even more, the addition of variant exons can define new glycosylation sites; e.g., in the case of exon v3 that contains a Ser–Gly–Ser–Gly motif, which acts as a binding site for both chondroitin sulfate (CS) and heparan sulfate (HS), promoting a differential interaction of CD44 with the microenvironment (Yamano et al. 2016). For example, as mentioned above, CD44v3 binds HS (but not CD44s) and through it, this isoform can bind HGF/ FGF2/ HB-EGF. In turn, these factors interact with their receptor tyrosine kinase, generating a complex for inducing a specific intracellular signal (Williams et al. 2013). CD44 integrates signaling in normal stem cells and cancer stem cells as well as the premetastatic niches (Suvorov et al. 2018). Thus, several antecedents indicate that CD44 and CD44v regulate differentially stem cell (normal and CSC) behavior within the niche through similar and exclusive mechanisms.

10.3.2 Hyaluronan

Hyaluronan (HA) is one of the central components of the ECM of vertebrates. HA has a simple covalent structure, which consists of alternating β-D-glucuronate and *N*-acetyl-β-D-glucosamine sugars (Hascall 2019). HA plays an essential role in tissue

homeostasis through its interaction with other ECM components. Besides facilitating the organization of the tissue, HA acts as an important modulator of cell behavior by triggering different cell signals. HA can interact with different cellular receptors (CD44, RHAMM, TLR4, LYVE-1), proteins (HABP, TSG-6), and proteoglycans. These interactions allow HA to modulate several cell processes including growth, adhesion, migration, and differentiation (Spinelli et al. 2015).

The properties of HA are directly dependent on its molecular weight. At homeostasis, HA of high-molecular weight (HMW, ranging from 0.5×10^6 to 2×10^6 Da) is predominant, having mainly hydrodynamic and structural properties. Interactions between HMW HA and other components of the ECM play an important role in ECM structural organization as well as certain signals, whereas the low-molecular weight (LMW, ranging from 10^4 to 0.5×10^6 Da) form is present mainly during inflammation. The degradation of HMW HA to LMW HA fragments often leads to the generation of biologically active oligosaccharides with different properties and functions (Noble 2002). HA metabolism is a balance between its synthesis and degradation. However, a dysregulated ECM leads to abnormal cell behavior and failure of tissue homeostasis and functions.

HA is synthesized by HA synthases (HASs) and is fragmented into bioactive molecules by hyaluronidases (HYALs) or reactive oxygen or nitrogen species (ROS, NOS) (Heldin et al. 2013). HASs are plasma membrane proteins that synthesize HA chains of various molecular weights on the inner side of the plasma membrane, which are then secreted to the ECM (Itano et al. 1999; Weigel et al. 1997). In mammals, HA is synthesized by three different isoenzymes named HAS 1, 2, and 3 (Vigetti et al. 2014). Specifically, HAS 1 and HAS 2 have been reported to produce HMW HA (200–2000 kDa), whereas HAS 3 is responsible for smaller fragments of HA (100–1000 kDa) (Itano and Kimata 2002; Itano et al. 1999). Hyaluronan fragmentation in tissues depends on the expression of Hyals and the damage of ROS/NOS species. On the one hand, Hyals are enzymes that cleave HA with high affinity (Jiang et al. 2011; Stern et al. 2006). Although Hyal1 and Hyal2 can catalyze degradation by cleaving β-(1,4) linkages, Hyal1 degrades HA into small fragments (hexasaccharides and tetrasaccharides) whereas Hyal2 appears to produce larger fragments of approximately 20 kDa (Schwertfeger et al. 2015). On the other hand, HA is also fragmented by radicals of ROS/NOS species, especially in inflamed tissues (Schwertfeger et al. 2015).

The direct role of HA in regulating HSC in the niche is well documented, and its synthesis is essential after ablation by irradiation (Nilsson et al. 2003). HA is synthesized in stromal cells and by primitive hemopoietic progenitor cells (linages) in murine and human CD34+ BM progenitors, and its synthesis decreases when the cells mature (Darzynkiewicz and Balazs 2012). Moreover, HA synthesis, specifically by HAS3, allows the homing and lodging of hematopoietic stem cells. It was observed in HAS-3–/– knockout mice that lack of HAS3 leads to aberrant distribution of the grafted cells in bone marrow compared to the wild-type recipients (Ellis et al. 2011).

10.4 CD44–HA Interaction in the Stromal Cell from the Stem Cell Niche

10.4.1 Biological Functions of CD44 Associated with Stemness Properties in the Niche

The interaction between HA and CD44 through the amino-terminal region of the extracellular domain of CD44 generates conformational alterations that favor the binding of adaptor molecules to the cytoplasmic tail of this receptor. As a consequence, HA binding activates specific cell signaling pathways, including MAPK, Ras, and PI3K/Akt (Lv et al. 2016), which subsequently increases cell adhesion, migration, and proliferation (Ponta et al. 2003). The capacity of CD44 to bind HA is tightly regulated and cell-type specific.

Furthermore, it is in relation to the activation state of CD44 since this receptor can be present in inactive or active forms, depending on whether or not CD44 is able to bind the specific ligand (Lesley et al. 2000). It has been demonstrated that alternative splicing, changes in glycosylation, clustering, and modulation by the cytoplasmic tail can all determine transformation between inactive and active forms of CD44 (Isacke and Yarwood 2002). In addition to being the primary receptor for HA, CD44 is able to interact with other ECM components and acting as a co-receptor for different molecules that can modulate cell behavior.

10.4.2 CD44 as an ECM Molecules-Binding Protein

As mentioned above, alternative splicing during translation of the CD44 gene can insert binding sites for other GAGs from the ECM. Particularly, CD44v3 isoform can be substituted with HS chains transforming it into a heparan sulfate proteoglycan (HSPG) (Bennett et al. 1995) and allowing the interaction with heparin-binding growth factors such as bFGF. Even more, CS modified CD44 can bind fibronectin and collagen (Faassen et al. 1992).

On the other hand, the CD44 receptor can mediate important cell-signaling events, through the interaction with other molecules, such as matrix metalloproteinases (MMPs) enzymes and different growth factors. The association between different MMPs and receptors (such as CD44) involved in cell adhesion may be important in mediating pericellular degradation of the ECM to facilitate cell migration. Several studies demonstrated that CD44 acts as a modulator of secretion and activation of MMP-2 (Takahashi et al. 1999). Besides, it has been shown that CD44 can recruit and anchor the active form of MMP-9 and interact with MT1-MMP (Yu and Stamenkovic 2000; Mori et al. 2002). HA–CD44 interaction is essential for the co-clustering of CD44 and MMP-9 since this ECM-degrading enzyme binds to CD44 in a different binding motif, which is available after HA binding (Bourguignon et al. 1998). Once co-clustered, CD44 and proteolytically

active MMP-9 isoform can induce the degradation of collagen IV, promoting tumor cell migration. Besides, the interaction between CD44 and MMP-9 activates TGF-beta through the proteolytic cleavage of inactive TGF-beta, which activates neovascularization and angiogenesis (Yu and Stamenkovic 2000). Importantly, these interactions between MMPs and CD44 are affecting the stem cells mobilization (Klein et al. 2015)

HA–CD44 interaction regulates stem cell migration and homing in different stem cell niches. CD44 has a crucial role in the arresting and rolling of leukocytes through HA chains present on the ECM of the endothelium through the upregulation of VCAM-1 and ICAM-1 proteins on endothelial cells. Indeed, the interaction between the cytoplasmic tail of CD44 and integrin α4β1 in T cells is required to promote their specific adhesion to the endothelium and allow the extravasation into inflammatory sites in vivo (Bertoni et al. 2018).

Several studies have shown that a specific glycoform of CD44, HCELL, expressed on primitive CD34+ hematopoietic progenitor cells promotes homing in the bone marrow favoring rolling interactions on E-selectin expressed on bone marrow-endothelial cells (Dimitroff et al. 2001). Moreover, CD44–HA interaction regulates trans-endothelial migration and anchorage in bone marrow through the action of stromal cell-derived factor 1 (SDF-1). Specifically, SDF-1 promotes hematopoietic progenitor cell adhesion to HA, and the formation of actin-containing protrusions with CD44 (Avigdor et al. 2004).

10.4.3 CD44 as a Cell Surface Co-receptor

The variant isoforms of CD44 can function as co-receptors of many cell surface molecules and growth factors.

CD44 also can function as a co-receptor by binding and anchoring growth factors on the cell surface, to allow the presentation and the stabilization to their specific receptors. Specifically, it was demonstrated that CD44v6 has an extracellular peptide domain that binds to hepatocyte growth factor (HGF), the ligand for the c-Met receptor. In tumor cells, CD44v6 is necessary for HGF signaling since it is responsible for the formation of a complex between HGF and c-Met, which finally activates c-Met by the interaction with the cytoplasmic tail of CD44v6 (Orian-Rousseau 2010). Furthermore, these results were confirmed using neutralizing antibodies to CD44v6 and studying a tumor cell line in which only CD44s and c-Met receptors were expressed. In both cases, it was not possible to induce the activation of the c-Met receptor, which confirms the key role of CD44v6 in the activation of HGF signaling (Orian-Rousseau et al. 2002).

The interaction between CD44 and HGF has also been demonstrated in several types of cells. In primary keratinocytes, HGF-induced Met activation depends completely on the presence of CD44-v6 isoform. Nevertheless, HS modification of CD44 is not required for this synergy between mature HGF, Met, and CD44-v6 proteins (Orian-Rousseau et al. 2002). It has also been proposed that a subpopulation

of hematopoietic stem cells responds to HFG–CD44 interaction by migrating toward skeletal muscles (Rosu-Myles et al. 2005). Finally, HGF promotes the enhancement of endothelial cell barrier functions via CD44 isoforms. Specifically, it was observed that HGF promoted c-Met association with CD44v10 and recruitment of c-Met into caveolin-enriched domains containing CD44s (Singleton et al. 2007).

On the other hand, it was discovered that CD44v6 and vascular endothelial growth factor receptor 2 (VEGFR-2) interact constituting a complex detected by co-immune precipitation. Even more, a CD44v6-specific peptide, as well as a CD44v6 neutralizing antibody, reversed VEGF-induced VEGFR-2 activation and downstream ERK activation (Tremmel et al. 2009). CD44 acts also as a co-receptor for the ERbB receptor tyrosine kinase family, specifically with ERbB1 (HER1), ERbB2 (HER2), and ERbB3 (HER3), which was demonstrated to be crucial for the activation of CD44 receptor in carcinoma cell lines. Furthermore, silencing of CD44v6 decreased ErbB2 phosphorylation in colon cancer cells, suggesting that CD44v6 is involved in the activation of this oncogenic pathway (Misra et al. 2009).

CD44v6 can also modulate transcription of the HGF gene, which stimulates the growth of different cell types such as fibroblasts, endothelial cells, and vascular smooth muscle cells. Even more, HGF promotes the recruitment of mesenchymal stem cells to different niches (Zhang et al. 2006). Even more, it has been discovered that another isoform of CD44 receptor, CD44v3, binds basic fibroblast growth factor (bFGF) that stimulates proliferation of underlying mesenchymal (Sherman et al. 1998; Lee et al. 2009).

10.4.4 CD44 as a Modulator of ABC Transporter Function

ATP-binding cassette (ABC) transporters are proteins localized in cell membranes involved in the active excretion of several molecules and cell metabolites. ABC transporters superfamily have an important role in the maintenance of tissue integrity due to a broad spectrum of physiological activities; including detoxification, selective permeability, lipid metabolism, defense against oxidative stress and xenobiotics, as well as the development of drug resistance in most types of tumors (Russel et al. 2002; Szakacs et al. 2008; Leslie et al. 2005). ABC transporters are highly expressed in hematopoietic stem cells compared to other progenitor cells. The expression of ABC transporters assures life-long protection of hematopoietic stem cells and preserve stem cell integrity by extrusion of agents that trigger their differentiation (Tang et al. 2010). Moreover, two specific ABC transporters, the multidrug resistance gene 1 product (MDR1/ABCB1) also called P-glycoprotein (P-gp), and ABCG2, have been involved in tissue regeneration. Both efflux pumps are highly expressed on a particular and small subpopulation of cells called side population formed by CD44+ primitive bone marrow-derived stem cells with long-term repopulating abilities. The loss of expression of ABC pumps, as well as CD44 receptors, leads to cell differentiation. Side population cells have been implicated in the regeneration of various organs, such as the liver, skeletal muscles, and heart

(Huls et al. 2009) as well as in the development of chemotherapy resistance (Wang et al. 2015; Hou et al. 2018).

Multiple causes, in which CD44 can be implicated as a cell-adhesion molecule as well as an HA receptor can cause the development of chemotherapy resistance in a tumorigenic stem niche. One of the main mechanisms, which promotes tumor reappearance, can be mediated by the upregulation of the expression of several drug transporters such as MDR-1, which is encoded by the multidrug resistance (MDR) gene. As a consequence of an increase in MDR-1 expression, tumor cells can diminish drug uptake or induce higher drug efflux out of the cells. Even more, it has been demonstrated that, in breast cancer cells, the interaction between HA and CD44 produced an increase in MDR-1 expression, promoting tumor cell proliferation and survival (Bourguignon et al. 2009). The physical and genetic interaction between CD44s and MDR-1 transporter has been investigated for analyzing the relation between both molecules, which is, in part responsible for the invasive potential in cancer cells. The expression of CD44 in sensitive tumor cells promoted the expression of MDR-1. Furthermore, specific-MDR-1 substrates that interfere with its function reduced invasion, migration, and the physical co-localization of CD44s and MDR-1 in vitro. These data demonstrated that CD44s and MDR-1 closely and physically interact, which results in the concurrent expression and modulation of invasion and drug resistance phenotypes (Miletti-Gonzalez et al. 2005).

10.4.5 CD44 as a Modulator of Hypoxia

Local oxygen concentration is shown to be an important factor in stem cell niches. It can directly influence stem cell self-renewal and differentiation. Some studies suggest that hypoxia provides a suitable niche for stem cells to maintain their precursor status. One hypothesis proposes that stem cells might benefit from residing in hypoxic niches, where oxidative DNA damage may be reduced (Liang et al. 2017; Keith and Simon 2007). Cipolleschi et al. have shown that the deeply hypoxic areas in the bone marrow niche seem to be suitable for the long-term maintenance of stem cells, while the better-oxygenated areas allow the proliferation of more differentiated progenitors (Cipolleschi et al. 1993). Also, hypoxia induces different cellular adaptations during tumorigenesis and progression of tumor, such as a switch to anaerobic metabolism, increase in angiogenesis, genetic instability, promotion of invasiveness, and stemness preservation (Zhu et al. 2017). In hypoxic conditions, hypoxia-inducible factor 1 (HIF-1) is stabilized and activated. HIF-1 is a major transcription factor that detects low O_2 levels and induces adaptive cellular programming for hypoxic conditions. The expression of HIF-1α is much dependent on oxygen levels. While in normoxia, HIF-1α is continuously expressed and degraded; in hypoxia, its degradation is prevented. Furthermore, it was shown that long-lasting hypoxia boosts the production of reactive oxygen species (ROS), which activates HIF-1α transcription. HIF-1 helps cell survival by activating the transcription of proto-oncogenes, growth factors and angiogenic factors, metastasis, and drug resistance

and therefore it has a major role in tumorigenesis (Konopleva and Jordan 2011; Vera-Ramirez et al. 2011).

Some reports show that cancer stem cells (CSCs) have the ability to migrate to areas of injury, which are considered to be areas of hypoxia and necrosis. This process, according to some studies, appears to be driven by SDF-1 and plays a significant role in angiogenesis and metastasis (Vera-Ramirez et al. 2011). It has been shown that hypoxia and ROS induce CSCs to express HIF-1α, directly promoting EMT (Plaks et al. 2015). Many regulatory molecules that are involved in EMT, including HIF-1, are associated with the metabolic reprogramming of cancer cells. The induction of EMT is implicated in the acquirement of CSC properties, reduced mitochondrial metabolism, and stimulation of the glycolytic switch (Lee et al. 2017).

The nuclear factor NF-κB is a transcription factor that binds to κB sequences in DNA and activates the expression of more than 200 genes associated with inflammation, cell proliferation, and differentiation. Several of these genes are involved in the carcinogenic process. It has been shown that H_2O_2 or hypoxia may cause its activation, although this is not the standard mechanism by which NF-κB is activated (Vera-Ramirez et al. 2011). The upregulation of NF-kB promotes cancer cell stemness. Furthermore, transforming growth factor-beta (TGF-β) can also directly interact with NF-kB signaling pathways to additionally increase stemness (Plaks et al. 2015). NF-kB was implicated as a regulator of CD44 expression in melanocytes. Some studies show that CD44 expression in hepatoma and cervical cancer cells was regulated via NF-kB binding in the promoter of the CD44 gene (Smith et al. 2014).

HAS2 has been associated with tumor progression in different types of cancer. Zhu et al. have shown that there is a direct binding of both HIF-1α and NF-κB on the promoter of the hyaluronan synthase two antisense 1 (HAS2-AS1) gene in oral squamous cell carcinoma (OSCC) cell line. They suggest that hypoxia-induced HAS2-AS1 expression is dependent on both HIF-1α and NF-κB binding directly to the HAS2-AS1 promoter and activating it (Zhu et al. 2017). HAS2-AS1 can regulate the expression of HAS-2 and is considered to be necessary for the induction of the transcription of the HAS2 gene (Vigetti et al. 2014; Neill and Zoeller 2015).

There is an upregulation of both CD44 and hyaluronan (HA) under hypoxic conditions. CD44–HA interaction has been implicated in different tumor-related cell signaling events, such as the promotion of anchorage-independent tumor cell growth, migration, and metastasis. The upregulation of CD44 and HA under hypoxic conditions could magnify these events. Additionally, Krishnamachary et al. have established the role of HIF-1α in regulating CD44 in MDA-MB-231 and SUM-149 breast cancer cells (Krishnamachary et al. 2012).

The progression of solid tumors is frequently being associated with hypoxia. However, several reports show that the progression of leukemia in a rat model was associated with marked expansion of hypoxia (Konopleva and Jordan 2011). Also, it has been shown that after chemotherapy, surviving cells might display EMT and CSC phenotypes and oncogenic metabolism. It was suggested that the hypoxic regions of tumors could provide protection for CSCs, and increase their survival

during chemotherapy (Lee et al. 2017). Taken together all these reports highlight the importance of hypoxic areas in tumorigenesis.

10.4.6 CD44 as a Modulator of Migration and Anchorage

The migration of stem cells is an essential process during organogenesis and development but also for tissue homeostasis and repair after injury. It has been shown that CD44 and HA have an important role in cell migration for a variety of normal and malignant cells. Avigdor et al. have shown that CD44 affects human hematopoietic stem/progenitor cells trafficking to the bone marrow by interactions with HA. Matrix HA supports cell adhesion and regulates cell trafficking in the bone marrow niche where it is produced by stromal and hematopoietic cells (Avigdor et al. 2004). The influence of CD44 and its interaction with HA on cell migration were also shown in other biologic processes, such as inflammation and tumor metastasis. In the process of tumor metastasis, tumor cells need to acquire new adhesion and migration properties, mainly a new set of adhesion, migration, and homing proteins. This process requires, to a significant part, the modulation of cell–ECM adhesion and cell–cell adhesion (Jothy 2003).

Hematopoietic stem cells reside in the bone marrow niche. However, these cells can leave this niche, enter the peripheral circulation, and travel to different tissues where they can divide and differentiate. Hematopoietic stem/progenitor cells can also undergo the process of homing into the bone marrow. Homing of these stem cells into their niches requires tightly regulated interactions between the progenitors and the microenvironment. It has been suggested that CD44 has a pivotal role in the adhesion of the hematopoietic stem cells to the HA of bone marrow ECM (Hess and Allan 2011; Avigdor et al. 2004). CD44 is one of the cell adhesion proteins that is specialized in both cell–cell and cell–ECM adhesion. This trait makes the dysregulation of its expression possibly involved in the invasive and metastatic potential of tumor cells (Jothy 2003). In some tumors, HA production is upregulated in response to tumorigenic conditions. One of the proposed mechanisms of cell adhesion and migration in these tumors is classical HA–CD44 interaction: CD44-positive cancer cells adhere to the HA-rich ECM on the endothelial surface or tissue stroma. It was shown that serum-derived HA-binding protein (SHAP) in complex with HA induced visibly stronger cell adhesion (Itano et al. 2008).

Stromal cell-derived factor-1 (SDF-1) or CXCL12 is a chemokine that has been usually associated with two chemokine receptors, CXCR4 and CXCR7, which are able to activate different downstream signaling pathways. CXCR4 is mostly associated with cancer invasion. The CXCL12/CXCR4 pathway was also implicated in recruiting stem cells from the bone marrow to the sites of injury to help in tissue regeneration (De-Colle et al. 2018; Liepelt and Tacke 2016). It was shown that SDF-1 induces the adhesion of human hematopoietic stem/progenitor cells to HA which is expressed in the bone marrow endothelium and causes CD44 distribution to the migrating hematopoietic stem/progenitor cells. The proximity of HA and

SDF-1 at the bone marrow endothelium further facilitates migration and anchorage of these cells to their niche (Avigdor et al. 2004).

On the other hand, SDF-1α can interact with different ECM components such as heparan sulfates or hyaluronan. By modulating its interaction with ECM SDF-1α is assumed to be able to increase the survival of cancer cells in bone marrow. Some reports indicate that HA interacts cooperatively with SDF-1α in directing cell motility through CD44 and CXCR4 receptor signaling crosstalk (Purcell et al. 2012). Liu et al. have also shown the existence of crosstalk between SDF-1α and the hyaluronan receptor CD44 and that both CXCR4 and CD44 drive in a Rac1-dependent manner, cell migration (Liu et al. 2017).

10.4.7 CD44 as a Modulator of Quiescence

As previously stated, quiescence, as well as other properties such as self-renewal, differentiation, and survival, are functions of the niche which interacts with residing stem cells. Quiescence is, in essence, a stem cell feature, allowing them to "rest" for a time until signal integration directs them to proliferate and differentiate. CD44 standard and CD44 variant isoforms have been implicated in quiescence maintenance (Zoller 2015). The BM niche where HSCs reside has stromal acellular components such as collagen and HA, among others. These are produced by stromal cells such as MSCs. MSCs support hematopoiesis meaning that they have an active role in maintaining quiescence also, thus keeping tissue homeostasis. The matrix secretory capacity in BM-MSCs supports stem cell tissue anchorage via CD44, inhibiting cell proliferation. The hypoxic niche within the bone marrow promotes the survival of stem cells, being normal (MSCs, HSCs) or dormant tumor cells.

10.4.8 Cellular Components of the Niche Stroma

Stromal support cells or "niche cells" are cells that are located close to stem cells. They are specific to a type of stem niche; however, common cells could be associated with the stem cells themselves. Among those cells, MSCs and the cells derived from them, like fibroblasts, osteoblasts/osteocytes, and adipocytes, and immune cells (macrophages) are increasingly recognized as components of stem cell niches (Reinwald et al. 2016).

10.4.8.1 MSCs

MSCs have multipotential differentiation capacity and although they were initially described in the BM, they have been isolated from multiple tissues, such as adipose tissue and, blood and umbilical cord, among others. This population of cells and

their progeny are the main organizers of the hematopoietic niche. These precursor cells could be differentiated from BM hematopoietic cells by their ability to quickly adhere to plastic (Domenech 2016). Besides, these cells have multipotent differentiation capacity and could give rise both in vivo and in vitro to mesenchymal tissues such as adipose, cartilaginous, and bone tissue. MSCs, like every stem cell, have an asymmetric division pattern, a fundamental characteristic of stem cells, since this allows them to maintain a stable pool of cellular elements with different degrees of differentiation (Karnoub 2017).

MSCs multipotentiality is provided by the combined expression of transcriptional regulators associated with pluripotential states, such as Sox2, Nanog, and OCT4, which act in an interconnected manner. Sox2 transcription factor is required to maintain self-renewal and the state of undifferentiation of osteoblast precursors and MSCs. The expression of this factor is diminished during osteoblastic differentiation, which allows the activation of the Wnt signaling pathway, which has pro-osteogenic effects (Seo et al. 2013). There are currently three minimum criteria to define the MSCs, proposed by the ISCT (International Society for Cellular Therapy) that are: (1) MSCs must adhere to plastic and form colonies (CFU-F) when grown under standard conditions; (2) they must express 95–99% of CD105, CD73, and CD90, and should not express CD34, CD45, CD14 or CD11b, CD79a or CD19 and HLA-DR (MHC); (3) They must be able to differentiate into osteoblasts, chondroblasts, and adipocytes (Dominici et al. 2006). Considering its easy isolation, its capacity for multipotent differentiation, and the ability to incorporate into growing tumors such as OS (Bolontrade et al. 2012), MSCs have emerged as possible useful tools for clinical applications in the area of regenerative medicine (Horwitz et al. 2006). Another striking feature of the MSCs is that they have an innate tropism toward sites of injury, moving from sites such as the BM into sites of inflammation, in response to various chemotactic signals (Mishra et al. 2009; Spaeth et al. 2009). Even when CD44 is not listed as one of the minimally necessary expressed markers in defining MSCs (Dominici et al. 2006), it is one of the surface markers that define BM-MSCs as well as MSCs from other niches. The presence of CD44 in MSCs has been related to migratory properties (Zhu et al. 2006). Related to this and in connection to migratory abilities, CD44-positive MSCs was demonstrated to be involved in binding to E-selectin and thus anchorage to activated endothelial cells, a necessary step in cells migrating from distant tissues (Ruster et al. 2006) Further, ECM remodeling is critical for cell migration by providing a permissive matrix. In this line, MMP9, one of the metalloproteinases secreted during tumor and normal tissue remodeling, has been demonstrated to bind to CD44, promoting the degradation of collagen by activated fibroblasts and thus allowing tumor cell invasion (Yu and Stamenkovic 1999).

10.4.8.2 Immune Cells

Immune cells within the stem cell niche are specific resident cells or recruited after a state of inflammation or injury. Hematopoietic stem cells (HSCs) are multipotent

cells that generate cells of all blood lineages. These cells reside in a specialized microenvironment in the bone marrow (BM), the HSCs niche that crucially regulates HSCs survival and function. It is well known that MSCs and endothelial cells are key regulators of the maintenance of the HSCs niche. However, there are other cell types, like immune cells, that contribute to it (Riether et al. 2015). Even more, the BM serves as a primary and secondary lymphoid organ, hosting various mature immune cells, including T and B cells, dendritic cells (DCs), macrophages, among others (Mercier et al. 2011). These immune cells provide a niche involved in the regulation of homeostasis in the HSCs niche.

Activated immune cells are able to bind HA and their response depends on several factors, such as the kind of cell, the molecular weight of HA, and the HA-binding proteins present in the tumor milieu (Spinelli et al. 2015, 2019). HA–CD44 interactions have an important role in inflammation, like immune cell recruitment, migration, adherence, chemotaxis, and phagocytosis/endocytosis (Spinelli et al. 2015). It was previously reported that CD44 is expressed on HSC and has been implicated in the homing of these cells after bone marrow transplantation (Lee-Sayer et al. 2015; Sackstein 2004; Avigdor et al. 2004) and there is evidence that HA is a component of the stem cell niche in the BM (Haylock and Nilsson 2006; Protin et al. 1999). However, CD44 null mice usually develop (Lee-Sayer et al. 2015; Protin et al. 1999).

Macrophages are a heterogeneous population of cells, which show a high degree of functional plasticity. Macrophages have the ability to integrate diverse signals from the microenvironment and adopt different phenotypes: M1 (classically activated) and M2 (alternatively activated) (Ruffell and Coussens 2015). An in vitro study showed that the expression of CD44 is polarization dependent: (1) CD44v6 was found exclusively in M2 macrophages and (2) the overall CD44 amount was higher in M1 than M2 (Rios de la Rosa et al. 2017). Moreover, it was previously reported that macrophages can maintain HSCs quiescence, retention in the niche and their depletion causes HSC mobilization (Chow et al. 2011; Winkler et al. 2010; Ludin et al. 2012). A study showed that M1-macrophages can inhibit HSCs self-renewal, whereas M2-macrophages can promote it (Luo et al. 2018). Even more, it was found that macrophage depletion induced the HSCs niche to collapse leading to HSCs mobilization from the BM to the blood (Kaur et al. 2017; Chow et al. 2011).

In contrast, the tumor microenvironment creates an isolated niche for tissue-resident macrophages. Tumor-associated macrophages (TAMs) are M2-like macrophages and, in most cases, play an immunosuppressive role and work in concert with tumor cells, promoting growth and progression (Mantovani et al. 2017). It was previously shown that a reduction in the number of TAMs can decrease the number of Cancer Stem Cells (CSCs) in pancreatic tumors. Whereas in glioma, the distribution of TAMs in the marginal area correlated with the location of CD133+ glioma cells (Ye et al. 2012). Interestingly, CSCs are the major subset that can render macrophages with the ability to facilitate the production of tumorigenic factors like IL-6 (Jinushi et al. 2012).

10.5 CD44–HA Interaction in Tumor Transformation

ECM perturbations may cause stem cell dysfunctions and alterations in the niche, promoting neoplastic transformation. On the other hand, the malignant processes might alter the normal stem cell niche. One example was observed in myeloma cells that disrupt the endosteal HSC niche by secreting the Dkk1 a Wnt inhibitor (Qiang et al. 2008). Besides, the normal niche can attract malignant cells with similar characteristics to the stem cells of the niche. It has been observed in bone marrow, for example for osteotropic cancer cells, they could migrate and extravasate from the peripheral circulation into perivascular regions through adhesion molecule that includes integrins and CD44 (Hess and Allan 2011).

The ECM contributes also to the cell transition state that in turn contributes to transition niches. The different cellular populations, as mentioned above, are interacting within these niches, and their secretome impact in the neighboring populations and may determine or induce a switch of phenotype. Cells possess the ability to transit among morphologies by a change in their phenotype which allows them to transition between well-defined states. One of these transition processes is the epithelial to mesenchymal transition (EMT), where CD44 and AH expression have been shown to be associated with, contributing to metastasis in the malignant process (Chen et al. 2018). During EMT, cells convert from an epithelial phenotype characterized by tight cell–cell and cell–basement membrane interactions, cell polarity, and E-cadherin expression, into a mesenchymal phenotype. The transition event involves the expression of key genes involved in regulating the EMT process (Snail, Twist, and Zeb1) and the disruption of cell–cell and cell–matrix interactions leading to the loss of cell polarity and cytoskeleton remodeling, improving motility and secretory capacities. The resulting cell presents a mesenchymal phenotype with the capacity to migrate through the secretion of MMPs (MMP2, MMP9, MT1-MMP) involved in the degradation of the basement membrane, cells also express proteins such as N-cadherin, α-smooth muscle actin (αSMA), fibronectin, vimentin, and desmin, and secrete different components of the ECM. EMT represents a biological mechanism involved in remodeling processes, like wound-healing, embryogenesis and development, fibrosis and cancer. In the tumoral context, EMT is the process that allows epithelial-like cells from a primary tumor, to shift from a phenotype that involves anchorage-dependent survival and cell polarity into one that enables cell migration toward future metastatic sites. Tumoral EMT process is not only related to augmented motility, but it is also linked to a gain of stemness, immune evasion, and multidrug resistance (Lu and Kang 2019).

On the other hand, the reverse transition process named mesenchymal to epithelial transition (MET) represents a cell switch from a mesenchymal-like phenotype into an epithelial-morphology. As opposed to the previous transition, MET involves the downregulation of the activated mesenchymal-associated genes like N-cadherin, fibronectin, and others, mediated by epithelial-associated proteins such as E-cadherin itself, ELF5, and others. EMT/MET processes are both implicated in tumoral progression and cells transit through these programs at different times and in

different niches. EMT is necessary for the acquisition of many metastatic traits, which allows cell colonization of new microenvironments that would become metastatic sites. Conversely, once a cell reaches a premetastatic niche, the MET process is activated, allowing metastatic cells to walk back into their steps and adopt an epithelial-like phenotype, consolidating the secondary tumor growth site or metastatic focus (Lu and Kang 2019).

The cellular and acellular components of the different cellular niches have the ability to regulate both EMT and MET processes through the secretion of different signaling molecules that activate downstream pathways involved in the regulation of these biological processes. Several antecedents showed that CD44 should be critical in MSCs transformation into TAFs (Tumor-associated fibroblasts), pointing that the presence of CD44 would not only regulate anchorage to a residence tissue, but would permit adequate modulation to leave the niche of residence, enter the circulation, and integrate signals to adapt and respond to the new environment (Spaeth et al. 2013).

Besides, it has been observed that also HA expression plays a central role in EMT. It has been demonstrated that overexpression of HAS2, that synthetizes HA, is associated with an E-cadherin downregulation, activation of β-catenin, expression of Snail, Twist, and Zeb, transcriptional molecules that drive EMT (Zoltan-Jones et al. 2003; Koyama et al. 2007). Moreover, HAS2 expression is associated with EMT induces by TGF-β (Porsch et al. 2013).

10.6 Conclusion

Biochemical interactions between the stem cell and its niche can determine their respective functions, and CD44–HA is one of the main interactions that regulates multiple functions, especially in a tumor context. Therefore, the stem cells and their acellular niche, the extracellular matrix, should be analyzed as compartments that interact dynamically. The ubiquitous cellular receptor CD44 and its main ligand HA have a prominent role in the interactions between the stem cell, the niche, and the transition states, determining cell fate and, when hemostasis is functioning, allowing tumor transformation. Thus, the present perspective in this area is to obtain a better understanding of the role of these interactions in the transformation of normal stem niches to a tumorigenic niche that in turn allows the development of cancer stem cells. This knowledge could be beneficial for the advancement of new therapeutic approaches that target these cancer stem cells.

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Chapter 11
Proteoglycans in Glioma Stem Cells

Florian A. Siebzehnrubl

Abstract Proteoglycans are important biomolecules in development, injury, and disease. They are highly prevalent in the central nervous system, where they are components of the extracellular matrix or expressed on the cell surface to contribute to the regulation of cell signaling, cell adhesion, and cell–matrix interactions. Expression of proteoglycan core proteins and key synthetic or degradation enzymes is aberrant in brain cancers, including gliomas. Some proteoglycans, such as CD44 or CSPG4/NG2, have been implicated as cell-surface markers of stem/progenitor cells in the brain. Signaling through these proteoglycans is also promoting glioma progression and cancer stem cell maintenance. This book chapter will review the known functions of proteoglycans in glioma cancer stem cells.

11.1 Introduction

Proteoglycans are present in virtually all mammalian tissues. They are important components of the extracellular matrix (ECM) as well as co-regulators of signaling pathways, cell adhesion, and interactions between cells and their environment. Proteoglycans consist of a protein backbone, referred to as core protein, which is post-translationally modified by the addition of glycosaminoglycan (GAG) chains of varying lengths that contain further chemical modifications (e.g., phosphorylation, sulfation). The different structures of core proteins and modification of GAG chains create a highly diverse family of proteoglycans fine-tuned to fulfill a wide range of biological functions.

Proteoglycans are abundant in the central nervous system (CNS) and serve crucial functions during development, injury, and disease. Deletion or mutation of several proteoglycans result in neurodevelopmental disorders, underlining their importance for the development and function of the CNS (Conway et al. 2011a, b; Mclaughlin

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et al. 2003; Silver and Silver 2014). The chief functions of proteoglycans are to act as an extracellular reservoir for growth factors (when secreted) or as co-receptors for growth factor signaling pathways (when membrane-associated). These functions enable the formation of morphogen gradients, e.g., during development, which may also affect cancer progression and invasion. Additionally, proteoglycans contribute to interactions between a cell and its microenvironment. For instance, CSPGs provide repulsive signals for axon growth cones, generating boundaries between developing components of the brain, but also prevent axonal regrowth after spinal cord injury as part of the resulting glial scar (Tran et al. 2018; Silver and Miller 2004).

Proteoglycan biology is highly complex due to the great structural and functional diversity of these molecules. While the molecular functions of several proteoglycans and specific residues in their GAG motifs have been elucidated, many questions remain unanswered. Particularly in brain cancers, their contributions to tumor progression and malignancy are not fully understood. This chapter will highlight currently known functions of proteoglycans in gliomas, with a specific focus on their relevance for brain cancer stem cells.

11.2 Proteoglycan Family Members and Protein Structure

Proteoglycans consist of a core protein that is decorated with GAG side chains. Depending on the GAG chains, proteoglycans are classified as heparan sulfate (HSPGs), chondroitin sulfate (CSPGs), or keratan/dermatan sulfate proteoglycans. This chapter will focus on HSPGs and CSPGs, which are best characterized. Their core proteins can be used to subclassify HSPGs and CSPGs (Table 11.1). The proteoglycan core protein structure influences proteoglycan functions, such as whether they are membrane-associated or secreted into the extracellular space.

Proteoglycan core proteins can contain a transmembrane domain, a glycophosphatidylinositol (GPI)-anchoring site, or no membrane-associated domains. Thus, some proteoglycans contain both extra- and intracellular domains, while others are tethered to the extracellular membrane or secreted into the extracellular matrix. The core protein structure is, therefore, key to understanding the function of the proteoglycan. For instance, the transmembrane proteoglycan syndecan-1 (SDC1) modulates integrin signaling via its intracellular domain (Beauvais and Rapraeger 2010).

The CSPGs expressed in the CNS belong to the lectican subfamily and contain a conserved N-terminal and C-terminal globular domain which are linked by a backbone of varying length. CSPG core proteins range in length from 294 to 3396 amino acids (Silbert and Sugumaran 2002). CSPGs can be grouped as ECM or membrane-bound proteoglycans (Table 11.1). Extracellular CSPGs are an essential part of the brain parenchymal ECM, together with hyaluronic acid and other linker proteins. Examples of membrane-bound CSPGs are CD44 and NG2, which act as signaling co-receptors. In the CNS, CSPGs are well known for their repulsive functions on

Table 11.1 Representative members of HSPG and CSPG families. Shown are the size of core protein (amino acids), number and type of GAGs, as well as their localization as extracellular matrix (ECM), membrane-bound, or secreted proteoglycans

Family	Proteoglycan	Gene symbol	Core protein (aa)	GAGs	Localization
HSPG	Perlecan	HSPG2	3588, 4346	1–3 HS	ECM
	Agrin	AGRN	2045	1–3 HS	ECM
	Collagen XVIII	COL18A1	1336, 1516, 1751	2–3 HS	ECM
	Syndecan 1-4	SDC1-4	198, 201, 310, 443	1–3 HS/ 1–2 CS	Membrane-bound
	Glypican 1-6	GPC1-6	555–580	1–3 HS	Membrane-bound
	Serglycin	SRGN	158	10–15 heparin/CS	Secreted
CSPG	Aggrecan/ CSPG1	ACAN	2431	~100 CS	ECM
	Versican/ CSPG2	VCAN	655, 1642, 2409, 3396	12–15 CS	ECM
	Neurocan/ CSPG3	NCAN	1321	1–2 CS	ECM
	NG2/CSPG4	CSPG4	2322	2–3 CS	Membrane-bound
	Brevican/ CSPG7	BCAN	911	0–4 CS	ECM
	Phosphacan	PTPRZ1	1448, 1455, 2308, 2315	2–5 CS	Membrane-bound / ECM
	Decorin	DCN	359	1 CS	ECM
	Biglycan	BGN	368	1–2 CS	ECM
	CD44	CD44	294–493	1–4 CS	Membrane-bound

Modified from Masu (2016); Bulow and Hobert (2006)

axon growth during development and injury. In development, this guides the formation of axonal projections across the brain, whereas deposition of CSPGs in injury contributes to the formation of a glial scar that is prohibitory to axonal regeneration (Silver and Silver 2014).

HSPG core proteins range in length from 158 to 4346 amino acids and consist of three groups: membrane-associated, ECM, and secretory vesicle types (Table 11.1) (Bulow and Hobert 2006). The membrane-associated HSPGs include syndecans (SDCs; transmembrane proteins) and glypicans (GPCs; GPI-anchored), and act as co-receptors for growth factor signaling, protease receptors, and receptors for cell attachment (Esko et al. 2009). ECM HSPGs include perlecan, aggrin, and collagen XVIII, which provide an extracellular substrate for cell attachment and migration, as well as axon guidance molecules. In the CNS, ECM HSPGs are mostly part of the basal membrane around blood vessels and/or the pial surface. Lastly, serglycin comprises the secretory vesicle type (Esko et al. 2009).

The GAG chains decorating the core proteins are added and elongated through a complex biosynthetic pathway involving a large number of enzymes. GAGs form a

long, unbranched chain that is attached to serine residues in the core protein and always starts with a conserved sequence called tetrasaccharide linker (Silbert and Sugumaran 2002). The number of GAG chains on core proteins varies widely, ranging from 1 to >100 (Reviewed in (Masu 2016)). Some HS and CS sequence motifs have been well characterized structurally and biochemically, such as those binding to fibroblast growth factors (FGFs) (Xu and Esko 2014; Meneghetti et al. 2015, Mizumoto et al. 2015). We will discuss interactions between proteoglycans and FGFs in a later section.

GAG chain biosynthesis is initiated in the endoplasmic reticulum (ER) and happens simultaneous with the synthesis of the core proteins. The tetrasaccharide linker is added in the ER, whereas chain elongation is catalyzed in the Golgi apparatus. Chain elongation of GAGs is accomplished by alternate addition of monosaccharide residues through the cooperative and coordinated action of several different enzymes. While the tetrasaccharide linker is synthesized in the same way, chain elongation is notably different for CSPGs and HSPGs. CS-GAG backbone chains consist of *N*-acetylgalactosamine (GalNac) and glucuronic acid (GlcA), whereas HS-GAGs consist of *N*-acetylglucosamine (GlcNac) and GlcA (Masu 2016). CS-GAG chains are initiated by GalNAc transferase I and elongated by chondroitin synthases, chondroitin polymerizing factor, and CS *N*-acetylgalactosaminyltransferases (Masu 2016; Kwok et al. 2012). CS-GAGs are then modified, e.g., by phosphorylation and sulfation.

HS-GAG synthesis is initiated by exostosin-like-3, which adds GlcNac to the tetrasaccharide linker. Chains are elongated by the HS polymerase complex consisting of exostosins 1 and 2, adding alternating GlcA and GlcNac units (Xiong et al. 2014). The HS-GAG chain is then modified by a series of enzymes, starting with *N*-deacetylase/*N*-sulfotransferases 1-4, which deacetylate some GlcNac residues and convert them to glucosamine-*N*-sulfate. Then, glucuronyl C-5 epimerase converts some GlcA to Iduronic Acid (IdoA) residues. This is followed by several O-sulfation steps catalyzed by HS-2-*O*-sulfotransferase, HS-3-*O*-sulfotransferase, and HS-6-*O*-sulfotransferase. Finally, extracellular, postsynthetic modification (e.g., by endo-6-*O*-sulfatases or by heparanase, HPSE) can further increase the structural diversity of HSPGs (Xiong et al. 2014; Masu 2016).

The complex synthesis pathways enable the generation of highly diverse families of CSPGs and HSPGs that is mirrored in their functional diversity. The different functions of proteoglycans in gliomas and glioma stem cells will be discussed after a brief introduction to brain cancers in the next section.

11.3 Gliomas

The overwhelming majority of CNS tumors are glial in nature and are termed gliomas. Based on their histopathological appearance and molecular characteristics, gliomas can be divided into astrocytic (astrocytomas) or oligodendroglial (oligodendrogliomas) tumors (Deangelis 2001; Capper et al. 2018). Gliomas can

be either high-grade or low-grade. Low-grade astrocytomas can be divided into benign (e.g., pilocytic astrocytoma) and malignant lesions, whereas all oligodendrogliomas are malignant (Louis et al. 2016). Glioblastoma (GBM) is the only brain tumor of WHO grade IV and is the most frequent type of brain cancer in adults. GBM is the most malignant astrocytoma and can occur de novo (primary GBM) or arise from pre-existing lower-grade tumors (secondary GBM). Mutations in the *IDH1* gene (Toedt et al. 2011) distinguish primary (where they are absent) from secondary GBM (where they are present) and hint at a different origin and evolution of these entities.

Tumor grading is based on histopathological hallmarks, such as nuclear atypia, mitosis, vascular proliferation, and necrosis, and increasingly also considers molecular characteristics, such as loss of chromosomes 1p/19q and *IDH1* mutation status (Louis et al. 2016). Historically, gliomas have been classified based on their histopathological appearance, e.g., as astrocytomas or oligodendrogliomas, but more recent classification schemes also incorporate next-gen sequencing data and DNA methylation profiling (Capper et al. 2018). While the general glioma types are upheld in classification methods based on DNA methylation profiling and next-gen sequencing, these have revealed a much more diverse and heterogeneous nature of gliomas on the molecular level (Capper et al. 2018).

The glioma type has important implications for the prognosis of the tumor. For instance, oligodendrogliomas tend to respond much better to therapy, and the average survival of patients suffering from these tumors is longer than in astrocytomas (Van Den Bent et al. 2017). GBM, the only WHO grade IV brain cancer, is the most malignant brain tumor and also the most common in adults. GBM is incurable with a median survival of only 15–20 months with treatment. This is because GBMs initially respond to therapies, but over time become resistant and recur. Molecular profiling has revealed the rich heterogeneity of GBM, which may contribute to their therapeutic resistance. Gene expression profiling has demonstrated that GBM can be subclassified into multiple molecular subtypes, which frequently co-exist within the same tumor (Verhaak et al. 2010; Sottoriva et al. 2013; Neftel et al. 2019). GBM pathobiology is characterized by its diffusely invasive nature, high proliferation index, and propensity for neo-angiogenesis (Deangelis 2001). The histological appearance of GBM can vary widely, with high vascularization in some areas and others showing characteristic patterns of necrosis (Alexander and Cloughesy 2017). Necrotic areas within GBM are zones of hypoxia and associated with specific pathobiological processes (Colwell et al. 2017). Thus, the tumor microenvironment (TME) in GBM can be vastly different, and separate niches have been defined that impact tumor biology. These niches include the hypoxic, vascular, and invasive niche (Lathia et al. 2011).

All three niches have been found to provide a fertile environment for cancer stem cells, and it is likely that different factors in each niche promote cancer stem cell self-renewal through separate pathways. For instance, it was found that hypoxia induces cancer stemness through HIF1a and HIF2a (Li et al. 2009; Seidel et al. 2010), while VEGF signaling is active in the vascular niche (Gilbertson and Rich 2007). The invasive niche is less understood, but recent work has identified Notch signaling as

key pathway for enabling cancer stem cells to migrate along white matter tracts (Wang et al. 2019).

Cancer stem cells are capable of self-renewal, extensive proliferation, and initiation of tumor growth, whereas non-stem cancer cells are not (Lathia et al. 2015). Since their identification in GBM (Ignatova et al. 2002; Singh et al. 2003), the so-called cancer stem cell hypothesis has caused a major paradigm shift in cancer research and in our understanding of tumor development and progression. It is thought that cancer stem cells are at the apex of a hierarchy of tumor cells and are uniquely capable of initiating and promoting tumor growth (Vescovi et al. 2006). Akin to their normal counterparts, cancer stem cells can self-renew, producing another cancer stem cell and a non-stem cancer cell that lacks the ability to form new tumors. Their ability for extensive proliferation enables cancer stem cells to generate sufficient progenies to fuel the growth of a tumor. Cancer stem cells also have a much greater capacity to resist therapies, and therefore are likely culprits for treatment-refractive recurrence of GBM that results in their high lethality. Several transcriptional regulators have been identified that function to maintain the stem cell identity of cancer stem cells, including MYC (Wurdak et al. 2010; Chan et al. 2012), SOX2 (Gangemi et al. 2009), OLIG2 (Ligon et al. 2007), ZEB1 (Siebzehnrubl et al. 2013; Singh et al. 2017), STAT3 (Sherry et al. 2009), GLI1 (Clement et al. 2007), and others. These are activated by niche signaling pathways (Rheinbay et al. 2013; Day et al. 2013; Fan et al. 2010). Additionally, growth factor signaling, e.g., from EGF, PDGF, IL-6, TGF-b, and FGF2, maintains stemness in GBM (Kim et al. 2012, Wang et al. 2009, Jun et al. 2014, Ikushima et al. 2009, Jimenez-Pascual and Siebzehnrubl 2019; Jimenez-Pascual et al. 2019, Gargiulo et al. 2013). As described below, proteoglycans are important reservoirs for growth factor ligands in the extracellular space and co-regulators for the activation of their cognate receptors in brain cancer.

11.4 Proteoglycan Functions in Glioma

In glioma, proteoglycans form part of the ECM and the tumor microenvironment (TME). As in normal tissue, proteoglycans act as reservoir for growth factors, receptors for proteases, and co-receptors for signaling pathways. Thus, glioma cells may gain access to important pro-survival and pro-mitogenic factors through their release of proteolytic enzymes that cleave proteoglycans and release trophic factors into the TME (Kundu et al. 2016). Additionally, cleavage of GAG chains from HSPGs by the action of HPSE has been associated with increased angiogenesis and inflammation in other cancers (Vlodavsky et al. 2012). Aberrant cell-surface expression of proteoglycans may result in abnormal pathway activation that promotes survival and growth of glioma cells. For instance, upregulation of GPCs increases the sensitivity of cancer cells to growth factors, such as FGF2 (Su et al. 2006). Wade et al. (Wade et al. 2013) analyzed the prevalence of different HSPG and CSPG core proteins in GBM using publicly available gene expression datasets (i.e.,

from The Cancer Genome Atlas Project) and found differential regulation of several core proteins compared to normal brain tissue. For instance, GPC5 is downregulated in GBM, whereas GPC1 is upregulated. Among the CSPGs, CSPG4/NG2, PTPRZ1, CD44, as well as VCAN were upregulated. The specific functions of GPC1/5, PTPRZ1, and VCAN in glioma remain unclear. Some functions of CSPG4/NG2 and CD44 have been elucidated and will be discussed in the next section.

Alteration of proteoglycan core proteins and/or GAG chains may create a permissive environment for tumor growth and invasion, as well as silence the host response against the tumor. Silver et al. have shown that modification of CSPGs in malignant glioma affects the host response to the tumor and promotes tumor invasion (Silver et al. 2013). Crucially, this function is dependent on the depletion of CS-GAGs on the core proteins. If the core protein is glycosylated, host cells respond to the tumor with reactive gliosis and immune activation. This results in the formation of a glial scar that presents a boundary to infiltrating tumor cells, thus curtailing tumor invasion. Notably, this occurs only in the most benign gliomas. Conversely, de-glycosylation of the core proteins causes host cells to remain silent to the tumor and results in an absence of glial reactivity. This enables tumor cells to freely move throughout the neuropil and fosters invasion. De-glycosylated CSPGs seem to be a typical part of all invasive gliomas. Whether this is due to an active de-glycosylation of the core proteins or whether the biosynthesis pathways of CS-GAGs are compromised in these tumors remains to be determined. Wade et al. (2013) have found that the expression of several biosynthetic enzymes for both HSPGs and CSPGs is reduced in GBM, indicating that GAG chain structure and/or sulfation may differ crucially from healthy tissues. This implies that proteoglycan biosynthesis is compromised in glioma, but many other studies also indicate that proteolytic enzyme secretion is increased in malignant gliomas (Kundu et al. 2016; Jimenez-Pascual et al. 2019; Markovic et al. 2009). Aside from proteolytic digestion of the core proteins, extracellular enzymes that cleave GAG chains from HSPGs (e.g., HPSE) or change sulfation patterns (e.g., SULFs) are upregulated in gliomas, and this is correlated with patient survival (Wade et al. 2013; Kundu et al. 2016).

Proteolytic cleavage of proteoglycans and/or expression of structurally different proteoglycans by glioma cells may result in increased levels of trophic factors in the TME that create a permissive environment for the growth of cancer stem cells. The specific roles of proteoglycans in promoting glioma stemness are discussed in the next section.

11.5 Proteoglycan Functions in Glioma Stem Cells

Some CSPGs have been directly associated with stem/progenitor cells in development and cancer. The most notable examples include CD44 and NG2/CSPG4 (Pietras et al. 2014; Yadavilli et al. 2016). CD44 has been identified as a stem cell antigen in several tissues, including the brain (Zoller 2011). CD44 has also been identified as a marker on cancer stem cells in GBM (Pietras et al. 2014; Anido et al.

2010; Fu et al. 2013). CD44 is a receptor for hyaluronic acid, an ECM GAG that lacks a core protein and that is a key component of the ECM framework and thereby mediates cell adhesion to the ECM. Importantly, CD44 can signal to the nucleus and enhance HIF2a activity (Pietras et al. 2014). CD44 is therefore functionally involved in cancer stem cell maintenance by activating stemness-associated signaling pathways.

CSPG4 (more commonly known as neuron glia-antigen 2, NG2) was first identified to label a population of glial precursor cells that are capable of proliferating and generating oligodendrocytes and are therefore referred to as oligodendrocyte precursor cells (OPCs). Of note, OPCs are considered as a potential cell of origin for brain cancers (Liu et al. 2011). NG2 expression is increased in malignant glioma, including GBM, where it is associated with poor survival (Yadavilli et al. 2016; Svendsen et al. 2011). Whether NG2 expression is associated with a genuine cancer stem cell population is not fully resolved. It has been shown that NG2 knockdown results in slower glioma growth and reduced angiogenesis in vivo (Wang et al. 2011). A recent study reported NG2 expression in putative GBM cancer stem cells (Lama et al. 2016), but did not rigorously test whether isolating NG2 expressing GBM cells enriches for a cell population with higher tumorigenicity upon limiting-dilution orthotopic transplantation, which is the gold standard (Lathia et al. 2015). Nevertheless, this work showed that NG2 is also expressed on pericytes within the GBM core and at the invasion front, indicating that NG2 may also have crucial functions within the TME (Lama et al. 2016). NG2 can act as a signaling molecule, with its intracellular domain capable of binding extracellular regulated kinases (ERK1/2) and protein kinase C-alpha (PKC-a) (Ampofo et al. 2017). NG2 can thus activate cell migration, survival, and angiogenesis signaling pathways, all of which are relevant to tumor progression in malignant glioma.

To date, no HSPGs that are exclusive to, or enriched on, glioma stem cells have been identified, despite several HPSGs being upregulated in malignant glioma. Several studies have shown that FGFR1 is upregulated on GBM stem cells (Gouaze-Andersson et al. 2016; Jimenez-Pascual et al. 2019; Kowalski-Chauvel et al. 2019), and HSPGs act as co-receptors for FGF2-FGFR1 signaling. It is therefore possible that GBM stem cell-specific HSPGs or HPSG isoforms exist and will be identified in the future. Both CSPGs and HSPGs can bind FGFs and modulate FGF signaling (Guimond and Turnbull 1999; Allen and Rapraeger 2003; Djerbal et al. 2017). They may therefore be important co-regulators of cancer stem cell maintenance pathways, but these functions remain to be investigated.

As mentioned in the previous section, proteolytic cleavage of ECM proteoglycans may increase levels of bioavailable FGF2, e.g., to invasive glioma cells. Increased expression of membrane-bound CSPGs or HSPGs has been found in GBM (see above) (Wade et al. 2013). In glioma cells and glioma-associated blood vessels, aberrant expression of the HSPG GPC1 increases FGF2 sensitivity (Qiao et al. 2003; Su et al. 2006). Whether similar mechanisms exist on GBM cancer stem cells remains to be shown, but GPC1 is among the most prominently upregulated HSPGs in GBM (Wade et al. 2013), where it is predictive of invasion and poor prognosis (Saito et al. 2017). It is therefore conceivable that altered expression

and/or structure of CSPGs and/or HSPGs are key contributors to cancer stemness in glioma, but this remains to be shown.

11.6 Proteoglycans as Therapeutic Targets in Glioma

Because CSPGs and HSPGs are important co-regulators of receptor-tyrosine kinase signaling pathways dysregulated in glioma, they are candidate targets for anti-cancer therapies. Indeed, genetic depletion of CSPG4/NG2 was shown to reduce tumor growth in experimental models of glioma (Wang et al. 2011). The membrane-bound CSPG, CD44, has been used as a cell-surface marker for cancer stem cells, and it was shown that CD44 is functionally transducing signaling pathways on GBM cancer stem cells (Anido et al. 2010; Pietras et al. 2014).

As discussed above, posttranslational modification of proteoglycans is an important mechanism to generate their structural and functional diversity. It may therefore not be surprising that changes in posttranslational modification of HSPGs and CSPGs reflect on glioma malignancy. For instance, changes in HSPG sulfation are associated with more aggressive tumor growth, and knockdown studies of SULF2 resulted in decreased PDGFRa signaling and in vivo tumor growth (Phillips et al. 2012). Increased expression of the HSPG degrading enzyme, HPSE, is associated with reduced survival in GBM patients (Kundu et al. 2016). In experimental gliomas, it was found using syngeneic glioma transplants into HPSE-transgenic mice that host-derived HPSE contributes to tumor growth, immune evasion, and angiogenesis (Kundu et al. 2016). Knockdown approaches demonstrated that blocking expression of HPSE in pediatric glioma cells also decreases proliferation and invasion of these cells upon transplantation in vivo (Spyrou et al. 2017). The growth-promoting actions of HPSE with increased CD24 expression on GBM cells (Barash et al. 2019). For CSPGs, it was shown that de-glycosylation of CS core proteins is associated with increased invasion in GBM, whereas artificially increasing glycosylated CSPG levels in the ECM potently blocked tumor invasion (Silver et al. 2013).

All these studies implicate HSPGs and CSPGs as important contributors to glioma growth and malignancy. Because proteoglycans and certain proteoglycan-modifying enzymes reside in the extracellular domain, these constitute attractive targets for anti-cancer therapy. This has been evaluated in a number of studies using heparan sulfate mimetics, small-molecule inhibitors, and GAG antagonists. Heparan sulfate mimetics are sulfated oligosaccharides that can block HPSE and SULFs. They can scavenge ligands binding to HS side chains and may thus drain the TME of growth factors promoting glioma growth and progression (Johnstone et al. 2010; Dredge et al. 2011). One particular HS mimetic, M402, has shown promising results in experimental models of other solid tissue malignancies (Joyce et al. 2005) and is in clinical trials for pancreatic cancer. Additional inhibitors of HPSE have been developed (e.g., PG545), which block the release of biologically active GAGs from HSPGs in the TME (Hammond et al. 2013). In experimental models of glioma,

PG545 was shown to induce apoptosis in glioma cells, to reduce invasion, and to attenuate tumor growth in vivo (Kundu et al. 2016; Spyrou et al. 2017).

A recent study using a small-molecule sulfated GAG antagonist (Surfen) found that this molecule blocked CSPG receptor expression on glioma cells and decreased tumor invasion (Logun et al. 2019). While no specific inhibitors for the CSPG CD44 exist, this molecule is cleaved by gamma secretase for intracellular signaling, and gamma-secretase inhibitors showed promising results in experimental studies (Tanaka et al. 2015).

11.7 Summary and Conclusions

The functions of proteoglycans in glioma in general and glioma cancer stem cells, in particular, remain incompletely understood. The rich structural diversity of these molecules results in a wide range of functions that are fine-tuned according to biological needs. In brain cancer, it is becoming apparent that proteoglycan core protein expression, GAG synthesis, posttranslational modification, and extracellular structure are dissimilar to the normal brain. This diverse portfolio of potential structural changes of proteoglycans indicates that these molecules are important contributors to glioma growth and cancer stem cell maintenance.

A number of questions remain unanswered. Firstly, spatiotemporal heterogeneity of core protein expression and/or glycation is not fully understood. Many studies have investigated the HSPGs and CSPGs in glioma, but whether expression and/or glycation of these molecules changes in different areas of the tumor, or over time, is unclear. Secondly, the relationship between proteoglycan-modifying enzymes and cancer stem cells has not been properly addressed. HPSE and SULFs are capable of dramatically altering the structure of HSPGs, but the impact of this on cancer stemness is not understood. It is conceivable that extracellular modifications of HSPGs (and/or CSPGs) result in local changes of cytokine levels in the microenvironment that may promote stemness in glioma cells. Expression of HPSE and SULFs, too, may be subject to topological or temporal changes. Thirdly, whether proteoglycans act as signaling co-receptors and glioma cells and/or glioma stem cells has been only partially resolved. Whether certain cell-surface HSPGs or CSPGs are expressed preferentially on glioma cancer stem cells remains unclear, with the exception of CD44, where a firm relationship has been established, and potentially NG2, where expression of GBM cancer stem cells has been suggested. Cell-surface expression of proteoglycans on cancer stem cells likely results in an increased sensitivity of these cells to extracellular mitogens that enable cancer stem cells to thrive and populate tumor-free tissue. It will therefore be interesting to explore GBM cancer stem cell-specific expression of proteoglycans and their functions in the future.

The link of proteoglycans to pro-tumorigenic signaling pathways and tumor invasion also highlights their potential as possible therapeutic targets in glioma. Some studies have tested proteoglycan blocking agents in experimental models of

glioma (Kundu et al. 2016; Phillips et al. 2012), and some of these compounds are even in clinical trials for other solid tissue malignancies. Whether any promising compounds are able to cross the blood-brain-barrier, a major obstacle in drug delivery to brain cancers will need to be evaluated. Nevertheless, there is genuine potential for a new class of anti-cancer therapeutics aimed at the TME and at barring access of glioma cells to essential mitogens.

In summary, much more research needs to be done to unlock the potential functions of proteoglycans in glioma and to exploit these for therapeutic targeting.

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Chapter 12
Role of Syndecan-1 in Cancer Stem Cells

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Abstract Syndecan-1 (CD138) is a cell surface heparan sulfate proteoglycan that is frequently misexpressed in cancer. Under physiological and pathological conditions, it is involved in the regulation of diverse processes, such as leukocyte recruitment, wound repair, angiogenesis, exosome formation, and epithelial-to-mesenchymal transition. Apart from its role as an adhesion molecule and a modulator of proteolysis, Syndecan-1 has a pivotal function as a co-receptor for multiple signal transduction pathways, including Wnt, hedgehog, FGF, and NF-kB/IL-6/JAK/STAT3 signaling. Notably, the activity of these pathways plays an important role in determining the functional state of cancer stem cells. This cell population shares several properties with stem cells, and has been linked to cancer recurrence due to its property of increased resistance to chemo- and radiation therapies. In this chapter, we summarize the current knowledge on the role of Syndecan-1 in cancer stem cell function. Due to its simultaneous involvement as an enhancer of multiple stemness-associated signaling pathways, Syndecan-1 emerges as an attractive target for novel therapeutic approaches, which could be utilized to overcome recurrence after an otherwise successful therapy.

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12.1 The Cancer Stem Cell Hypothesis

Stem cells are the driving force in human growth and development and they guarantee the maintenance of organ homeostasis (Haston et al. 2018). Wound healing processes, immune defense, menstrual cycles of the female reproductive system, intestinal regeneration, hematopoiesis, and many other processes cannot proceed without the immense regenerative potential of stem cells (Klimczak and Kozlowska 2016). External factors disturbing these processes can lead to side effects and serious diseases. Organ regeneration after therapeutic interventions has always to be seen in the context of stem cell activity (Ito 2016). This regenerative force resides also in tumors and the cancer stem cell (CSC) hypothesis provides a perfect framework to address most aspects of tumor plasticity. The competing proliferative hypothesis, where all tumor cells have clonogenic potential and the driving force for proliferation resides in genetic alterations, could not explain all these aspects (Scott et al. 2019). However, the current CSC concept is still under debate and development by integrating actual experimental developments in this field.

According to the CSC hypothesis, tumors bear analog to organs an intrinsic heterogeneity with morphologically and molecularly distinct cell types and concerning this, an explicit cell-type-specific therapy resistance (Alvarado-Ortiz et al. 2019). The tumor bulk is largely constituted of differentiated cells, however, some cells bear stem cell character. Most of these specialized cells proliferate with high frequency while others are dormant and do not contribute to tumor development; they constitute a kind of backup pool. Explicitly these CSCs have self-renewal potential, can divide asymmetrically, give rise to metastasis, and can reconstitute a tumor after otherwise successful therapy (Alvarado-Ortiz et al. 2019) (Fig. 12.1). Quiescent cells are rare and often reside in special micro-niches, which protect them and promote their pronounced therapy resistance. However, the detailed impact of the extracellular matrix on tumor plasticity and resistance is still an outstanding question (Brown et al. 2019). High selection pressure through local micro-niche peculiarities such as a hypoxic and nutrient-deprived environment and acidosis promotes the CSC direction and contributes to therapy resistance (Vander Linden and Corbet 2019). Thus, the extracellular matrix is not only a scaffold for tissue organization, but also a factor influencing malignancy by biophysical and biochemical cues (Pickup et al. 2014). The communication with a niche containing host cells and matrix-associated structural components as well as extracellular metabolites, cytokines, chemokines, and ligands protects them as well and lets them maintain cancer hallmarks and escape immune attacks (Espinoza-Sánchez and Götte 2020; Menon et al. 2019).

Lapidot et al. (1994) could for the first time identify CSCs in acute myeloid leukemia (AML)-patients, and those cells were able to induce AML in SCID mice only if they exhibited the CD34+ CD38– marker expression profile, which agrees with that of normal human adult hematopoietic stem cells. This led them to hypothesize that the CSCs arise by the leukemic transformation. Since then, thousands of

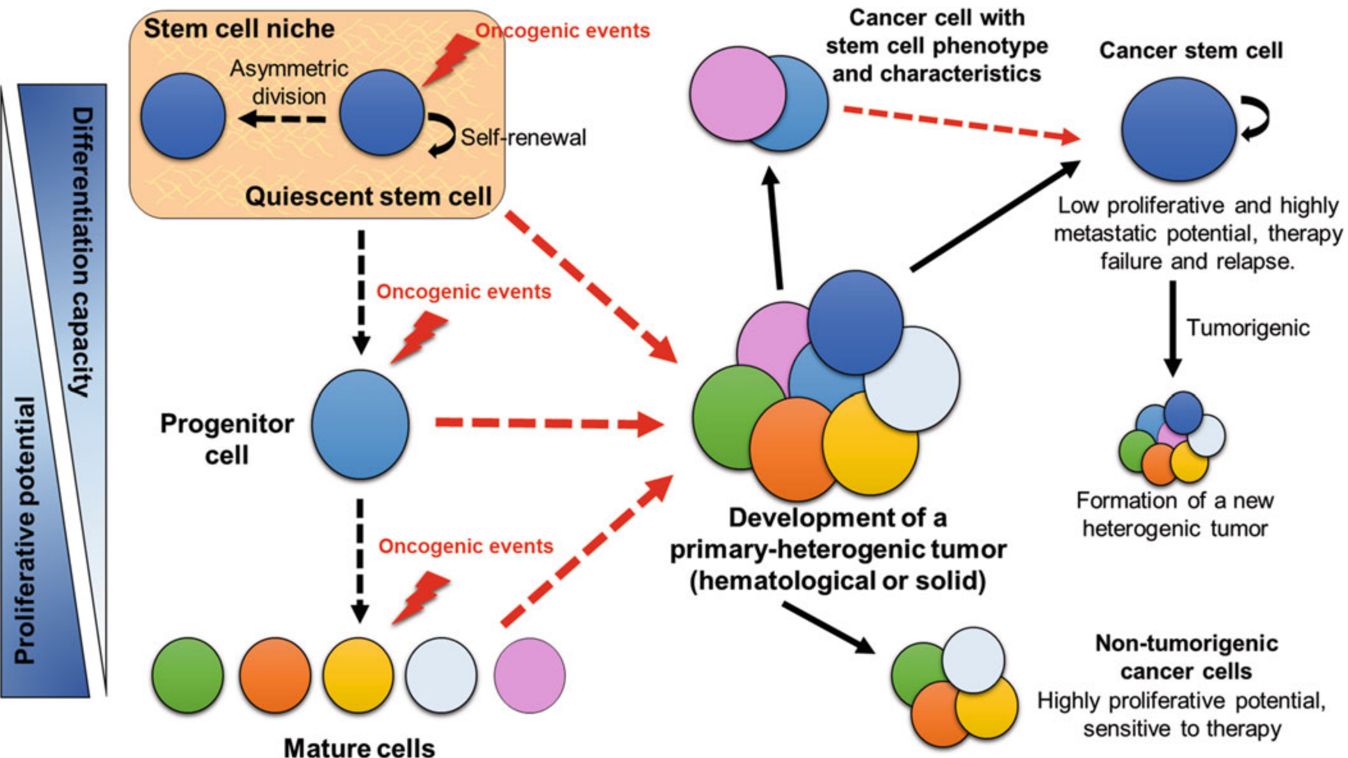

Fig. 12.1 The cancer stem cell hypothesis. Stem cells are defined by their capacity for self-renewal and differentiation toward progenitor cells and later toward multiple mature cell lineages. The capacity for self-renewal refers to a form of asymmetric division in which one of the daughter cells remains in a state of rest (quiescent) and the other commits itself to differentiation. Based on these characteristics, the hierarchical model suggests that if oncogenic events occur in normal stem cells, cancer stem cells (CSC) are generated, but if oncogenic events occur in mature or progenitor cells, tumor cells with stem characteristics are generated. Mature differentiated cancer cells form the heterogenic bulk of the tumor. Conventional antineoplastic treatment is directed to these highly

articles were published and the CSC aspect has emerged as an independent field of research.

What are the typical characteristics of CSCs? First of all, they can repeatedly constitute a xenograft tumor after the inoculation of a few cells (repeated adoptive transfer) (Lapidot et al. 1994). This was accomplished only if the cancer cells express a tumor entity-specific stem cell signature. The expression of CD44+ CD24–/low for breast cancer (Al-Hajj et al. 2003) or CD133+ for glioblastoma (Singh et al. 2004) match this profile and other marker combinations define CSCs for other tumors, like CD133+, CD44+, and CD117+ for prostate cancer (Harris and Kerr 2017), CD133+ and CD44+ for colon cancer (Kozovska et al. 2014), CD133+ for endometrial cancer (Ding et al. 2017; Götte et al. 2011) or CD133+ and CD44+, CD117+ and CD87+ in case of lung cancer (MacDonagh et al. 2016). Most of these CSC surface markers originate from embryonic and adult stem cell classification and possibly can be utilized for targeted cancer therapy. Besides marker expression, physiological features like export of xenobiotics by ATP-binding cassette (ABC) transporters (Fillmore and Kuperwasser 2008; Goodell et al. 1996), and strong activity of aldehyde dehydrogenases (ALDH) (Chute et al. 2006; Corti et al. 2006) are accepted CSC characteristics. These CSC-associated physiological peculiarities are directly related to therapy resistance, since the cell membrane located transporter system increases chemoresistance by exporting structurally foreign molecules like cytotoxic chemotherapeutics out of the cell, and cytosolic ALDH neutralizes reactive compounds after chemo- and radiation treatment and protects cells against oxidative stress (Greve et al. 2012). Human ALDH is comprised of 19 isotypes, which can be subdivided into 11 families and 4 subfamilies. They differ in substrate specificities and in their ability to metabolize aldehydes. In the stem cell field, ALDH1A1 and ALDH1A3 are considered the most important isoforms (Vassalli 2019).

Both stem cell features fulfill important functions in the maintenance of normal stem cells. The ATP-binding cassette transporters have protective potential and remove numerous substances like metabolic products, drugs, and toxic compounds, that could freely pass across the cell membrane. ALDH converts retinol to retinoic acids, which are involved in cell differentiation, and by this ALDH influences development and homeostasis of organs like the kidney and liver (Pierre-Louis et al. 2009; Greve et al. 2012). In addition to these CSC characteristics, many publications could show a more efficient DNA repair capacity, reactive oxygen and nitrogen species (ROS and NOS) scavenging, and sophisticated mechanisms to resist apoptosis and survive therapeutic interventions often under the influence of master developmental signaling pathways like the Notch-, hedgehog-, and Wnt-pathway, which further promote metastasis and cancer progression (Schulz et al. 2019; Vitale et al. 2019; Zhao et al. 2018). These signaling pathways become

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Fig. 12.1 (continued) proliferating non-stem tumor cells. The ability of CSCs to remain quiescent, together with a highly efficient DNA repair and drug expulsion machinery, enables CSCs to resist and survive drug treatments, favoring metastasis and eventually resulting in cancer relapse

modulated by specific ligands often mediated by cell surface heparan sulfate proteoglycans of the syndecan and glypican family (Vitale et al. 2019; Rai and Wang 2021; Jones et al. 2021), as outlined in the following section.

12.2 Cell-Surface Heparan Sulfate: A Versatile Integrator of Stemness-Related Signaling Events

Heparan sulfate is a carbohydrate of the glycosaminoglycan type that is attached to serine residues of core proteins bearing a consensus sequence of serine, glycine and one or more acidic residues through O-glycosylation in the Golgi apparatus (Karamanos et al. 2018). Synthesis of the linear, non-branched backbone of repetitive *N*-acetylglucosamine and β-D-glucuronic acid residues is followed by a series of partially incomplete enzymatic modification reactions including epimerization of the glucuronic acid, N-deacetylation, N-sulfation, and complex patterns of sulfation at 6-O-, 2-O-, and 3-O-positions (Rai and Wang 2021; Bowden and Nakato 2021; Teixeira and Götte 2020). As a result, the heparan sulfate chain is a highly anionic molecule under physiological conditions, which is organized in domains of varying degree of sulfation. It harbors both specific and more generic binding motifs for a multitude of ligands relevant to stem cell biology (Teixeira and Götte 2020; Vitale et al. 2019). As a constituent of cell surface, heparan sulfate proteoglycans of the syndecan-, and glypican families can influence stemness-related signaling events by either enhancing or inhibiting the formation and signaling through morphogen gradients, by modulating proteolytic activation of signaling receptors, and through a co-receptor function for receptor tyrosine kinases and heptahelical transmembrane receptors (Vitale et al. 2019; Karamanos et al. 2018) (Fig. 12.2). In the following sections, we will briefly highlight the importance of cell surface heparan sulfate for specific stemness-related signaling pathways. Further information can be found in additional chapters of this book (Rai and Wang 2021; Bowden and Nakato 2021; Siebzehnrubl 2021).

12.2.1 Wnt Signaling

Wnt ligands are a family of 19 secreted proteins implicated in the regulation of embryonic development and tissue homeostasis (Steinhart and Angers 2018). Distinct functions concerning proliferation and differentiation of stem cells, stem cell maintenance, or directional cell migration are determined by the interaction of these ligands with receptors of the canonical Wnt signaling pathway (frizzled and LRP5/6). These interactions result in the derepression of beta-catenin, or receptors mediating signaling through the noncanonical pathways of the planar cell polarity, receptor tyrosine kinase, and Ca^{2+}-pathways (Katoh 2017). There is ample evidence

for dysregulation of this signaling pathway in malignant disease and CSCs, and this knowledge has resulted in the development of inhibitors targeting Wnt ligands, receptors, beta-catenin, and Wnt secretion (Katoh 2017; Takebe et al. 2015). In CSCs utilizing the canonical pathway, engagement of the frizzled/LRP5/6 receptors by Wnt ligands released from cancer cells or cells of the tumor stroma results in protection of β-catenin and STOP target proteins such as YAP/TAZ, FOXM1, c-MYC, Cyclin D1, SMADs, and Snail from proteasomal degradation via a ubiquitinylation-dependent pathway. Nuclear translocation of stabilized beta-catenin subsequently results in the activation of genes under the control of TCF/LEF transcription factors, driving stemness-related gene expression (Katoh 2017). Data from model organisms indicated an important role for (cell surface) heparan sulfate in Wnt signaling, as several *Drosophila* mutants in genes affecting heparan sulfate biosynthesis (*sulfateless, sugarless*) and core proteins (*dally*, the fruit fly glypican) exhibited defects in signaling through the Wnt-related wingless pathway (Bernfield et al. 1999; Filmus 2001; Nybakken and Perrimon 2002; Bowden and Nakato 2021). Further evidence stems from studies on the frog *Xenopus,* where interactions between syndecan-4 and secreted R-spondin-3 induce Wnt-receptor complex endocytosis and activation of noncanonical Wnt signaling, whereas the interaction of syndecan-4 with fibronectin inhibited canonical Wnt signaling in a LRP6-dependent manner (Ohkawara et al. 2011; Astudillo et al. 2014). In the context of cancer pathophysiology, studies on the heparan sulfate sulfotransferase *HS3ST2* demonstrated that an increase in 3-O-sulfated heparan sulfate structures in human breast cancer cells of the aggressive triple-negative subtype resulted in an increased expression of the Wnt-dependent transcription factor TCF4 (TCF7L2), which was associated with increased invasiveness and cell motility (Vijaya Kumar et al. 2014). Moreover, overexpression of the heparan sulfate editing 6-*O*-endosulfatase SULF2 (which removes 6-*O* sulfate from *N*-glucosamine residues) in prostate cancer cells resulted in enhanced Wnt signaling and EMT, that was accompanied by increased cancer cell motility and proliferation (Vicente et al. 2015). Overall, these data provide evidence for the role of heparan sulfate as a modulator of Wnt signaling in development as well as malignant disease (Fig. 12.2).

The Wnt signaling pathway shows extensive crosstalk with additional stemness-related heparan sulfate-dependent signaling pathways (hedgehog, Notch, and FGF) that will be discussed in the following sections (Katoh 2017; Ranganathan et al. 2011).

12.2.2 The Hedgehog Pathway

Hedgehog signaling regulates epithelial-to-mesenchymal transition and tissue-patterning during embryonic development (Beachy et al. 2010; Clara et al. 2020). Mutations that lead to hyperactivation of this pathway have been observed in several tumor entities, including basal-cell carcinoma of the skin and medulloblastoma (Ng and Curran 2011). In CSCs, hedgehog activation promotes tumor growth, self-renewal and contributes to therapeutic resistance, as demonstrated for lung

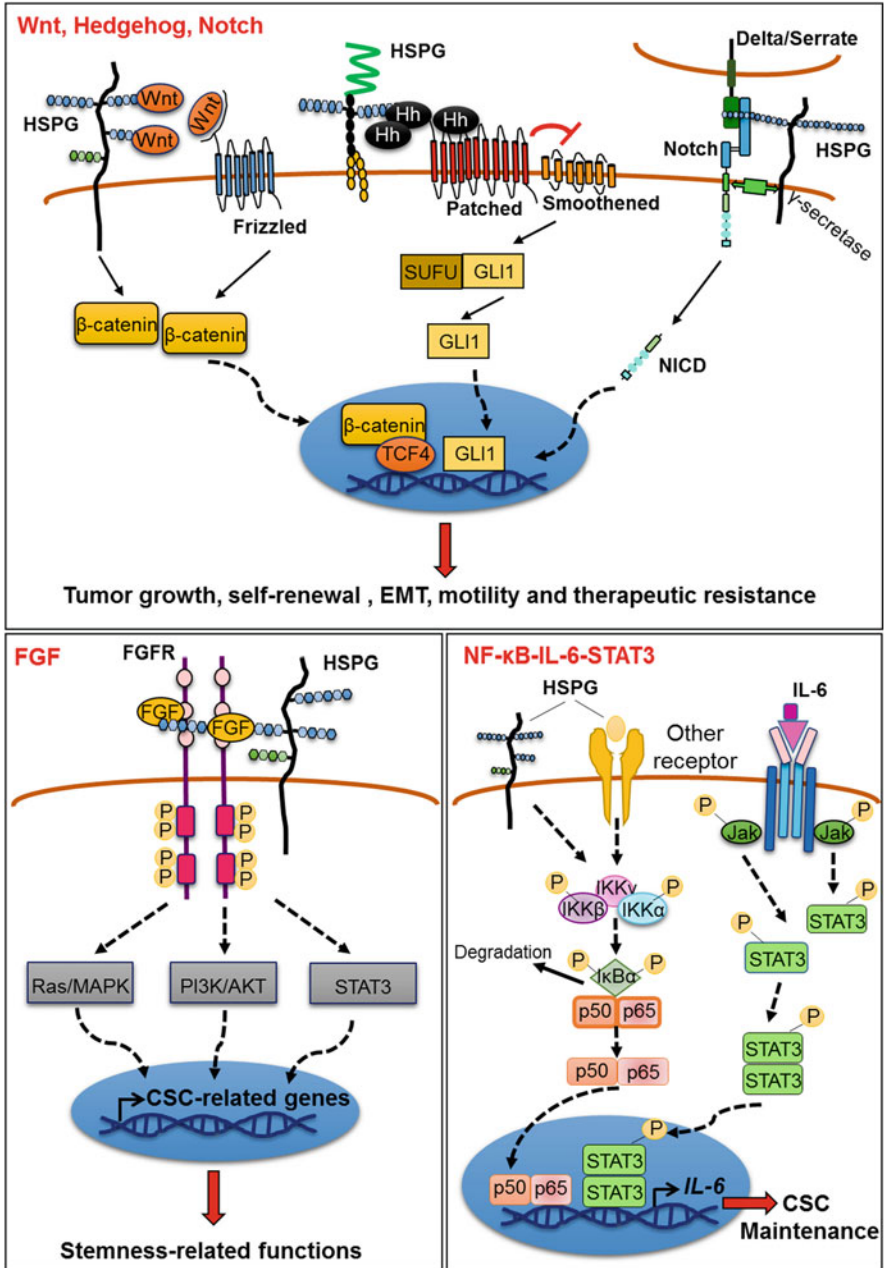

Fig. 12.2 Cell-surface heparan sulfate proteoglycans (HSPGs) influence stemness-related signaling pathways. Wnt, hedgehog, Notch, FGF, NF-κB, and IL-6/STAT3 are important pathways for normal development as well as for CSC induction and maintenance. Upper panel: As ligands or co-receptors, HSPG can activate and enhance the signaling through morphogen gradients inducing proteolytic activation of the receptors or acting together with tyrosine kinase receptors. In the Wnt signaling pathway, the desulfation of the heparan sulfate (HS) chains allows the presentation of Wnt ligands to Frizzled receptors. This interaction leads to the accumulation of β-catenin in the

and bladder CSCs (Po et al. 2017; Li et al. 2016). The hedgehog pathway is composed of the ligands sonic hedgehog, Indian hedgehog, and desert hedgehog, and a membrane-bound receptor system including the patched protein and the heptahelical transmembrane protein smoothened. Ligand engagement releases the inhibitory effect of patched on smoothened and results in the intracellular activation and nuclear translocation of Gli transcription factors, which in turn stimulate transcription of cyclin-dependent kinases and stemness-related factors such as Hes and Myc (Takebe et al. 2015; Clara et al. 2020) (Fig. 12.2). Of note, hedgehog signaling is functionally modulated by cell surface heparan sulfate, as evidenced by signaling defects in fruit flies and mice deficient in early-acting heparan sulfate biosynthetic enzymes such as EXT1, EXT2, and NDST (Karamanos et al. 2018; Teixeira and Götte 2020). Particularly the glypican family of glycosylphosphatidylinositol-anchored heparan sulfate proteoglycans plays an important role in regulating cellular responses to hedgehog gradients (Ortmann et al. 2015; Vitale et al. 2019; Shi et al. 2019). For example, 2-O-sulfated heparan sulfate motifs in glypican-5 were shown to promote neural progenitor cell proliferation via the sonic hedgehog pathway (Witt et al. 2013). While glypican-5 acted as a classical co-receptor in this context, an inhibitory function on this pathway has been assigned to other members of the family. For example, glypican-3, a proteoglycan that is co-expressed with stem cell markers in embryonic stem cells, rhabdoid, and germ cell tumors (Vitale et al. 2019) can compete with patched for hedgehog binding, resulting in a reduction of signaling (Capurro et al. 2015). Therefore, glypican-mediated modulation of

Fig. 12.2 (continued) cytoplasm which then translocates into the nucleus to activate TCF4. In the hedgehog pathway, the HS chains of HSPGs present Hh to Patched acting as a co-receptor and leading to the removal of smoothened. Subsequently, GLI1 is activated and separates from SUFU in the cytoplasm, and it subsequently translocates to the nucleus for the transcription of specific genes. In the Notch pathway, HSPG also acts as a co-receptor and even when the mechanism is not well understood it seems that the same sheddases that are responsible for cleavage and activation of Notch cleave and activate HSPGs. Also, the sulfated HS chains of the proteoglycans interfere with the activation of Notch. In this pathway, delta binds to Notch leading to the intracellular cleavage of the Notch intracellular domain (NICD) by γ-secretase. NICD translocates to the nucleus to regulate target gene transcription. Lower panel (left) In the FGF pathway, FGF is presented to FGFR at the cell surface in an HS-sulfation-specific manner. Through this interaction (FGF, HSPG, and FGFR) a ternary complex is formed, inducing the dimerization and phosphorylation of FGFR at its cytoplasmic domain. Its tyrosine kinase function activates other pathways such as Ras/MAPK, PI3K/AKT, and STAT3 to activate the expression of CSC-related genes. Lower panel (right) As a ligand or co-receptor, HSPG can activate the NF-κB and IL-6/STAT3 pathway. In the NF-κB pathway, the receptor activation induces the phosphorylation and subsequent degradation of IκB proteins (the NF-κB inhibitors). Upon IκB degradation, NF-κB (p50/p65) translocates to the nucleus to act as a transcription factor. One of the genes mostly expressed after activation of the NF-κB pathways is IL-6. Binding of the IL-6–IL-6R complex to gp130 phosphorylates JAK, which in turn phosphorylates STAT3 to become activated. The dimers of STAT3 translocate to the nucleus, upregulating the expression of IL-6 and other genes that contribute to CSC maintenance. Thus, the consequence of activating all these pathways in the cell is the induction of stemness-related functions and maintenance such as tumor growth, self-renewal, epithelial-to-mesenchymal transition (EMT) programs, which are associated with motility and metastasis, and resistance to chemotherapy

hedgehog signaling apparently depends on the family member involved. Further complexity is added by the observation that shedding of sonic hedgehog from the cell surface is enhanced in a cell autonomous manner depending on the heparan-sulfate chains of glypicans (Ortmann et al. 2015). In an oncological setting, a targeting of glypican-3 via chimeric antigen receptor (CAR)-T cells and immunotherapy in preclinical models has resulted in antitumoral effects, demonstrating the viability of this approach in overcoming CSC-mediated therapeutic resistance (Jiang et al. 2017; Espinoza-Sánchez and Götte 2020).

12.2.3 Notch Signaling

Like the aforementioned pathways, Notch signaling plays an important role in cell fate determination both during embryonic development and in CSCs (Clara et al. 2020; Troschel et al. 2020; Götte et al. 2011). However, in contrast to these pathways, Notch signaling relies on juxtacrine interactions of the four transmembrane receptors of the Notch family with membrane-bound ligands of the jagged and delta-like ligand (DLL) families on neighboring cells. Engagement of the receptors results in two subsequent proteolytic cleavage events, one extracellular step mediated by a disintegrin and metalloproteinase (ADAM), and a second intramembranous step mediated by gamma-secretase (Clara et al. 2020). As a result, the Notch intracellular domain is released, which interacts with nuclear factors and modulates stemness-related transcriptional programs in the nucleus (McIntyre et al. 2020) (Fig. 12.2). Initial results in the fruit fly *Drosophila melanogaster* indicated a role for 3-O-sulfated heparan sulfate in Notch signaling, as RNA interference-mediated downregulation of the heparan sulfate 3-*O*-sulfotransferase Hs3st-B function reduced Notch signaling and generated neurogenic phenotypes (Kamimura et al. 2004). However, more recently, these data have been controversially discussed, as a complete knockout of Hs3st-B did not affect Notch signaling, possibly suggesting a dosage effect of particular heparan sulfate modifications for Notch signaling (Guo et al. 2014). Notably, the Syndecan family of cell surface heparan sulfate proteoglycans apparently modulates signaling through the Notch pathway. For example, Notch-2 and Notch-3 mediated signaling regulate the levels of Syndecan-2 in smooth muscle cells, whereas syndecan-2 deficiency impairs Notch signaling in this system (Zhao et al. 2012). Moreover, in skeletal muscle, the satellite cell pool and the size of myofibers are apparently regulated via interactions of syndecan-3 with Notch (Pisconti et al. 2010), as discussed in detail by Jones et al. (2021). Furthermore, there is evidence for a functional interaction of Syndecan-1 with the Notch pathways in breast CSCs, as will be detailed in the next section of this chapter.

12.2.4 FGF Signaling

The fibroblast growth factor family comprises 22 members, many of which control important steps during embryonic development via signaling through their membrane-bound receptor tyrosine kinases (Rai and Wang 2021; Ornitz and Itoh 2015). Following ligand engagement, receptor dimerization, and autophosphorylation, several downstream signaling pathways are activated, including the Ras-MAPK, PI3K-Akt, PLCγ, and STAT pathways (Ornitz and Itoh 2015). Apart from the important role in coordinating developmental processes, an important function of this signaling pathway in stem cell biology is suggested by the observation that it is necessary to add FGFs to the culture media of human embryonic stem cells, neuronal stem cells, and CSCs in order to expand these cells (Ponti et al. 2005; Dvorak et al. 2006; Gotoh 2009; Haley and Kim 2014; Cocola et al. 2017). For example, FGF2 is required for self-renewal of colon cancer organoids, ensuring elevated expression levels of several self-renewal, and stemness-associated genes (Otte et al. 2019). Similarly, in glioblastoma CSCs, FGF2 induces expression of the stem cell marker nestin, while removal of FGF2 from the glioblastoma stem cell media results in cell differentiation (Pollard et al. 2009; Haley and Kim 2014; Chang et al. 2013). Moreover, activation of the FRS2 adaptor protein and its downstream signaling component Erk have been identified as important elements with respect to stemness-related functions (Gotoh 2009). Notably, the secreted canonical FGFs (including the Fgf1, Fgf4, Fgf7, Fgf8, and Fgf9 subfamily) bind to heparin in experimental settings, and to heparan sulfate at (stem) cell surfaces under physiological conditions (Ornitz and Itoh 2015). This interaction does not only lead to an enrichment of the FGFs at the cell surface that increases the probability of successful interaction with its cognate receptor(s), it does also allow for the formation of a ternary complex of heparan sulfate, FGF, and its receptor tyrosine kinase, thus facilitating signaling through this pathway (Sun et al. 2019a; Karamanos et al. 2018; Nikolova et al. 2009) (Fig. 12.2). Therefore, cell surface heparan sulfate proteoglycans are not only of relevance for FGF-signaling during embryonic development, but also in the context of CSC function (Bernfield et al. 1999; Karamanos et al. 2018; Vitale et al. 2019).

12.2.5 The NF-κB/IL-6/JAK/STAT3-Signaling Axis

The activation of inflammation-related signaling pathways plays an important role in tumor progression, as evidenced by the recent progress in the immunotherapy of malignant disease (Espinoza-Sánchez and Götte 2020). Indeed, dysregulation of pro-inflammatory pathways such as the nuclear factor kappa B (NF-kB) pathway or IL-6/JAK/STAT3 signaling has a direct impact on cancer patient survival (Espinoza-Sánchez et al. 2019; Johnson et al. 2018). Notably, it was shown that IL-6 and subsequent activation of STAT3 can promote the CSC phenotype in

different experimental systems (Ibrahim et al. 2013, 2017; Kim et al. 2013; Wang et al. 2016, 2018). The transcription factor NF-kB mediates proinflammatory gene expression and is a key regulator of progression from a chronic inflammatory state to cancer (Taniguchi and Karin 2018). One of the most strongly induced gene products is IL-6 (Brasier 2010). IL-6 can transduce signaling via different pathways. These include classical signaling after binding to the membrane-bound IL-6 receptor (IL-6R) present on restricted subsets of target cells, and the membrane-bound β-receptor glycoprotein-130 / gp130, as well as trans-signaling pathways which are mediated by soluble forms of the IL-6R (Johnson et al. 2018). The latter pathways reach larger cell populations owing to the more widespread expression of gp130 compared to the membrane-bound IL-6R (Rose-John 2018). In the classical pathway, initiation of signaling occurs following the formation of a heterohexameric complex of IL-6, IL-6R, and gp130. JAK proteins are now recruited and bind to Box 1 and Box 2 domains in the cytoplasmic part of gp130, resulting in tyrosine phosphorylation of the receptor and recruitment of STAT3. When bound to gp130, STAT3 is tyrosine phosphorylated by JAK allowing for STAT3 dimerization, nuclear translocation, and transcription of IL-6/JAK/STAT3-dependent target genes (Fig. 12.3). As these gene products include IL-6, this pathway is quickly regulated in a feed-forward manner. Trans-signaling involving soluble IL-6R occurs in a similar fashion (Johnson et al. 2018). Notably, IL-6 has been linked to CSC function, as IL-6-mediated activation of the JAK/STAT3 pathway has been observed in $CD126^{+}ALDH^{high}$ endometrial CSCs, and $CD44^{+}CD24^{-}$colorectal and breast CSCs (van der Zee et al. 2015; Marotta et al. 2011; Zhang et al. 2018). Notably, IL-6 promotes the conversion of breast cancer cells into a CSC-like state (Kim et al. 2013). As reviewed in detail by Bowden and Nakato (2021), heparan sulfate plays an important role as a modulator of JAK/STAT3 signaling in the fruit fly *Drosophila melanogaster*. Further evidence for a role of heparan sulfate in this pathway was provided by studies on the role of the heparan sulfate degrading enzyme heparanase in stem cell transplantation and graft-versus-host disease: Bone marrow transplantation experiments in mice indicated that stem cell engraftment was improved upon heparanase treatment, whereas only a mild manifestation of graft-versus-host disease was observed compared to a more severe phenotype in control mice (Bitan et al. 2010). Mechanistically, heparanase modulated the cytokine repertoire of T cells, including an increase in IL-6-production, which along with changes in other cytokines resulted in a reduction of T-lymphocyte activation. Recent studies in human ovarian carcinoma cell lines have expanded the concept of heparan sulfate modulation of the IL-6 pathway to human CSCs, as an inhibitory role of 3-O-sulfation of heparan sulfate (mediated by overexpression of the sulfotransferase HS3ST2) on IL-6-induced STAT3-signaling was shown (Huang et al. 2018). Moreover, ascorbate treatment of hepatocellular carcinoma in vitro and in vivo was shown to inhibit the activity of the heparan sulfate 6-*O*-sulfatase Sulf-2, resulting in a downregulation of IL-6 and NF-kB expressions, reduced cancer cell viability and prolonged survival of experimental animals (Alyoussef and Al-Gayyar 2018). Finally, as will be reviewed in the following section, original work from our laboratory and others has demonstrated a role for

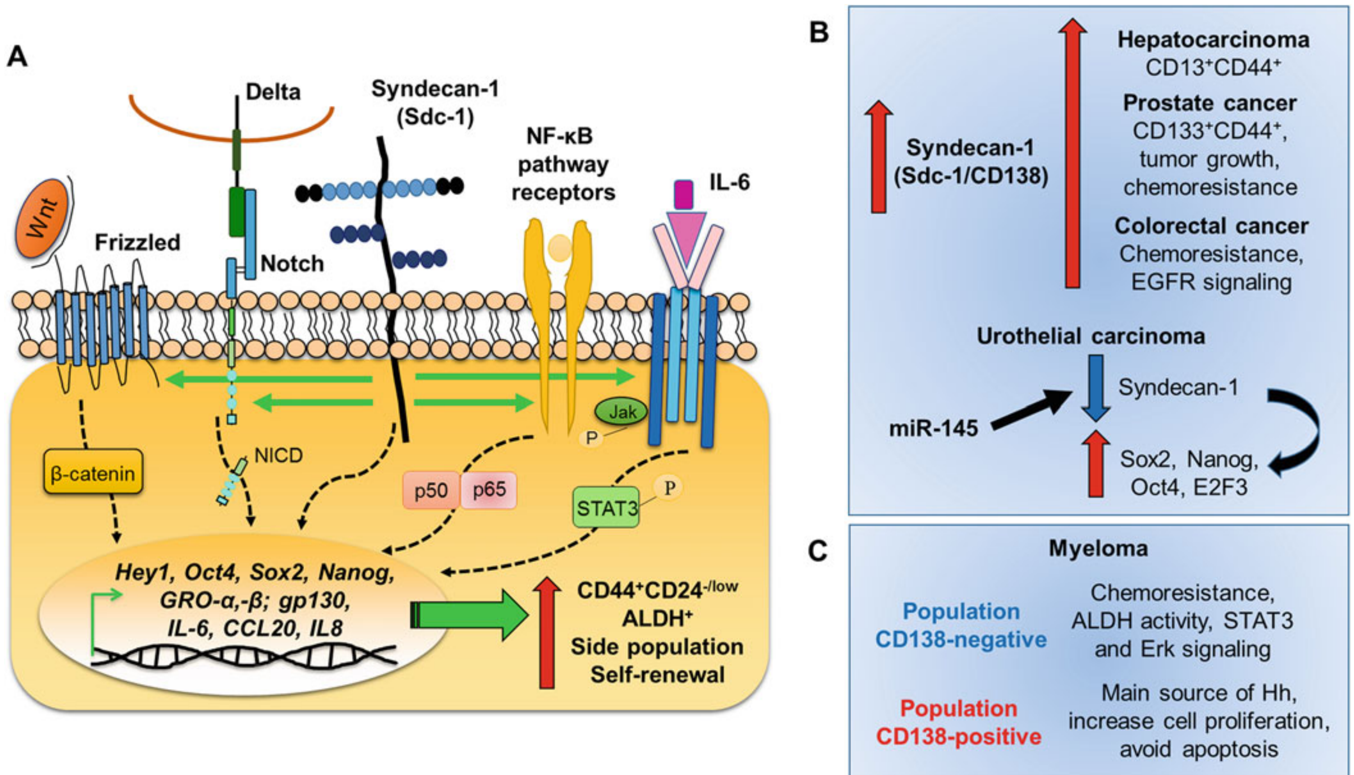

Fig. 12.3 The role of Syndecan-1 in signaling and functions of cancer stem cells (CSCs). (**a**) In breast cancer, Syndecan-1 (Sdc-1/CD138) increases CSC populations based on the expression of the following markers: CD24, CD44, and ALDH. Also, it increases the side population and self-renewal. The mechanism by which Sdc-1 operates is through the activation of different CSC-related pathways including Wnt, Notch, and IL-6/STAT3. NF-κB can be activated via signaling through multiple receptors, including toll-like receptors, tumor necrosis factor-alpha receptors, IL1 receptor, and CD40, among others. The activation of these pathways induces the expression of stem cell-related genes such as Hey, Oct4, Sox2, and Nanog and some factors associated with inflammation like

the heparan sulfate proteoglycan Syndecan-1 in IL-6-dependent signaling in inflammation-associated cancer and CSCs (Ibrahim et al. 2013, 2017; Binder Gallimidi et al. 2017; Hassan et al. 2013).

12.3 Syndecan-1: A Modulator of Cancer Stem Cell Function

Among the four members of the syndecan family of transmembrane heparan sulfate proteoglycans, Syndecan-1 is expressed earliest during embryonic development in the mouse (Bernfield et al. 1999; Götte 2003; Jones et al. 2021). During development and pathophysiological processes such as wound repair, it is transiently induced during the EMT, whereas in adults, it is highly expressed in epithelial and plasma cells (Bernfield et al. 1999; Jones et al. 2021). Structurally, Syndecan-1 is characterized by an extracellular domain harboring attachment sites for heparan sulfate and chondroitin sulfate glycosaminoglycan chains, as well as sites within the core protein that represent interaction sites for integrins and for cleavage of the ectodomain in a process known as shedding (Jones et al. 2021; Beauvais et al. 2009; Wang et al. 2005; Chen et al. 2008; Piperigkou et al. 2016). Syndecan-1 has well-documented functions as a binding partner for extracellular matrix molecules including fibronectin, laminin 332, and collagen isoforms, and as a modulator of numerous signaling pathways, including receptor tyrosine kinases, heptahelical G-protein coupled receptors, integrins, and Notch receptors (Bernfield et al. 1999; Götte 2003; Bachy et al. 2008; Gondelaud and Ricard-Blum 2019; Rapraeger et al. 2013; Ibrahim et al. 2017). Moreover, Syndecan-1 regulates the activity and expression of proteolytic enzymes and their endogenous inhibitors (Elenius et al. 2004; Vanhoutte et al. 2007; Nikolova et al. 2009; Ibrahim et al. 2012). Through this multitude of functional interactions, Syndecan-1 acts as a regulator of cell proliferation, adhesion, motility, and chemotaxis in numerous physiological and pathophysiological processes, including leukocyte recruitment during inflammation, wound repair, angiogenesis, and cancer metastasis (Götte et al. 2002; Kharabi Masouleh et al. 2009; Nikolova et al. 2009; Szatmári et al. 2015; Stepp et al. 2015; Teixeira and Götte 2020).

Fig. 12.3 (continued) chemokines (GROα/β and CCL20), receptors (gp130), and cytokines (IL-6 and IL-8). (**b**) In other cancers like hepatocarcinoma, prostate, and colorectal cancer, the high expression of Sdc-1 increases CSC populations associated with tumor growth and chemoresistance. In contrast, in urothelial carcinoma, the downregulation of Sdc-1 by miR-145 upregulates the expression of Sox2, Nanog, Oct4, and E2F3. (**c**) In myeloma, the population of CD138-negative cells is more associated with stemness characteristics, while the CD138-positive population is more related to mature cells, and shows high proliferation characteristics. However, some discrepancies exist regarding the function of CD138 in myeloma (see Sect. 12.3)

Besides the well-known functions of Syndecan-1, serving as a co-receptor for a multitude of growth factors, cytokines, and chemokines, we and others have shown that Syndecan-1 has a relevant role in regulating CSC functions. Our previous and recent data provide evidence that Syndecan-1 regulates cancer stemness in preclinical models and clinical tissue specimens of breast cancer (Ibrahim et al. 2013, 2017; Hassan et al. 2013). In the estrogen receptor-positive cell line MCF-7, showing a low metastatic potential, and the triple-negative, highly invasive cell line MDA-MB-231, siRNA-mediated Syndecan-1 silencing resulted in a significant reduction for the main CSC features, namely SP, ALDH-1, and the $CD44^{(+)}CD24^{(-/low)}$ phenotypes (Ibrahim et al. 2013). Functionally, Syndecan-1 depletion significantly retarded the formation of mammospheres in MCF-7 cells (Ibrahim et al. 2013). Several lines of mounting evidence indicate that CSC characteristics are mainly regulated by the same signaling pathways that govern embryonic development, including Wnt/β-catenin, Notch, IL-6/STAT3/NF-kB, and hedgehog signaling pathways (Clara et al. 2020; Vitale et al. 2019). Syndecan-1 affects the expression of the key signaling cues implicated in CSC regulation. For instance, we could demonstrate that Syndecan-1 knockdown not only significantly downregulated mRNA expression of IL-6, the IL-6 expression inducer chemokine CCL20 (MIP3α), and IL-6R, but also attenuated the constitutive activation status of the transcription factors STAT3 and NF-kB in MDA-MB-231 cells (Hassan et al. 2013) (Fig. 12.3). Another important signaling pathway driving breast CSC function is Wnt/β-catenin. In an attempt to decipher the role of Syndecan-1 in modulating Wnt signaling, Alexander et al. (2000) reported that Syndecan-1 knockout mice confer a reduction in Wnt-1-induced mammary carcinogenesis (Alexander et al. 2000). Furthermore, Syndecan-1 expression abrogation affected mammary development associated with the generation of mammary population resistant to Wnt -dependent tumor induction in the Syndecan-1 knockout mouse model (Liu et al. 2004). In an extension for their work, they further demonstrated that administration of the tumor-promoting agent 7,12-dimethylbenz[a]anthracene (DMBA) to Syndecan-1 knockout mice during juvenile development resulted not only in development of resistance to epithelial tumors, including liver and lung tumors but also to lymphoma (McDermott et al. 2007). We further confirmed the relevance of Syndecan-1 in this context for human breast cancer cells, as our in vitro data showed that the Wnt co-receptor LRP-6 protein was downregulated in Syndecan-1 knockdown MDA-MB-231 cells relative to controls (Ibrahim et al. 2013) (Fig. 12.3). This conforms to the role of LRP6 in CSC self-renewal and tumor growth as evidenced by its overexpression in human embryonic carcinoma cells (Dormeyer et al. 2008). Inflammatory breast cancer (IBC), a rare but highly aggressive and lethal variant of breast cancer, is enriched with CSC properties (Xiao et al. 2008, 2011). Therefore, in a further study, we verified our findings in the SUM-149 cell line as a representative IBC cell, and in Her2 (Neu/ErbB-2) positive SKBR3 cells, a representative for non-IBC. Both cells are rich in ALDH-1 activity, which was significantly reduced upon Syndecan-1 silencing (Ibrahim et al. 2017). Interestingly, employing immunohistochemical staining, we could show in this study a significantly higher expression of Syndecan-1 and the CSC surface marker CD44 in the triple-negative breast carcinoma tissues of IBC vs. non-IBC. Notably,

their expression had a strong positive correlation in carcinoma tissues of IBC (Ibrahim et al. 2017). Functionally, Syndecan-1 repression in SUM-149 cells disrupts 3D spheroid and colony formation. Mechanistic in vitro experiments revealed that Syndecan-1-specific siRNA treatment in both SKBR3 and SUM-149 cells downregulated the mRNA expression of CSC-related cytokines, chemokines, and their receptors/co-receptors, such as IL-6, IL-8, CCL20, and gp130.

In addition, Syndecan-1 expression had an influence on Notch signaling as Syndecan-1 suppression in SUM-149 cells repressed the mRNA expression levels of Notch-1, -3, -4, and the downstream target Hey-1 (Ibrahim et al. 2017). Intriguingly, a positive association between mRNA levels of Syndecan-1 and both Notch-1 and Notch-3 were observed in clinical tissue samples of IBC, but not in non-IBC, suggesting a functional association. Results of pharmacological Notch inhibition experiments in SUM-149 cells suggested that Syndecan-1 expression regulates the colony-forming ability and expression of inflammation-related cytokines via Notch signaling. At the protein level, the secretome of Syndecan-1-silenced SUM-149 cells was characterized by repressed levels of IL-6, IL-8, and growth regulated protein GRO-alpha, and GRO a/b/g relative to controls. Furthermore, expression of gp130, Notch-1, -2 proteins and the constitutively active status of STAT3 and NF-kB were attenuated upon Syndecan-1 abrogation (Ibrahim et al. 2017). A further study indicated that tumor cells present in the cerebrospinal fluid of patients with breast cancer leptomeningeal metastasis show elevated levels of Syndecan-1, MUC-1, and putative stem cell markers (e.g., CD44 and CD133), providing a link between a Syndecan-1 and CSC marker-positive cell population and brain metastasis (Cordone et al. 2017).

A role for Syndecan-1 as a regulator of CSC features was also demonstrated in other solid tumor types. In contrast to breast cancer, where a high expression of Syndecan-1 is associated with a poor prognosis, a reduction of Syndecan-1 expression is associated with a poor outcome in colon cancer, providing evidence for context-dependent functions of this proteoglycan (Teixeira and Götte 2020). Recent results from our laboratory showed that Syndecan-1 regulates colon CSC function via a focal adhesion kinase (FAK)–Wnt signaling axis (Kumar Katakam et al. 2020a). siRNA knockdown of Syndecan-1 in the human colon cancer cell lines Caco2 and HT-29 increased the side population (SP), enhanced aldehyde dehydrogenase-1 activity, and resulted in an upregulation of numerous stemness-associated markers including CD133, LGR5, EPCAM, NANOG, SRY (sex-determining region Y)-box 2, KLF2, and TCF4/TCF7L2. At the functional level, Syndecan-1 depletion increased sphere formation, and EMT-related gene expression. Notably, Syndecan-1-deficient cells showed increased resistance to doxorubicin chemotherapy and irradiation, which is consistent with their enhanced CSC properties. At the mechanistic level, an increased activation of β1-integrin, FAK, and Wnt could be linked to the enhanced CSC phenotype. In an extension of this work, we could demonstrate that a reduction of Syndecan-1 expression in Caco2 colon cancer cells resulted in an upregulation of the HS-degrading enzyme heparanase, via a mechanism involving upregulation and binding of the transcription

factor Egr1 to the HPSE promoter (Kumar Katakam et al. 2020b). Notably, plasmid-mediated upregulation of heparanase itself enhanced the stem cell properties of Caco2 cells, and was associated with increased resistance to radiotherapy, whereas high expression of an enzymatically inactive form of heparanase promoted chemoresistance to paclitaxel and cisplatin. Overall, our findings on the functional interplay of heparanase and Syndecan-1 in colon cancer may provide a novel concept for targeting stemness-associated signaling to overcome therapeutic resistance in this disease.

Apart from its role in colon CSCs, downregulation of *Sdc-1* gene expression diminished the gene expression of the CSC markers CD13 and CD44 associated with impairment of hepatoma sphere formation (Lin et al. 2020). Syndecan-1 is a downstream target for several microRNAs, mediating different molecular functions in pathophysiological diseases (Ibrahim et al. 2012, 2014; Fujii et al. 2015; Piperigkou et al. 2017). Overexpression of miR-145 promoted cell differentiation and senescence and upregulated stemness-related transcription factors, such as Oct4, Nanog, Sox2, and E2F3 in the human urothelial cancer cell lines T24 and KU7 via downregulation of Syndecan-1 (Fujii et al. 2015) (Fig. 12.3). Moreover, a vasculogenic/stem cell-like phenotype of melanoma patient cell lines and melanoma tumors was characterized by co-expression of Syndecan-1 with vascular mimicry markers, such as CD144 and VEGFR-2. In addition, a novel specific human recombinant antibody OC-46F2 raised against the Syndecan-1 extracellular domain impaired in vitro tubule-like structure formation (Orecchia et al. 2015). Syndecan-1 is a potential biomarker for CSCs in the AG2 colon cancer-initiating cell line (Suhovskih et al. 2015). In prostate cancer, stemness enriched-tumor initiating single cell-derived holoclones overexpress Syndecan-1, and stable RNA-mediated Syndecan-1 knockdown diminished their survival in vitro (Shimada et al. 2013). More importantly, Syndecan-1 silencing significantly reduced CD133/CD44-expressing cancer-initiating cells and tumor growth, and increased sensitization to docetaxel in vivo. Clinically, biochemical recurrence of prostate cancer was significantly associated with Syndecan-1 expression, confirming Syndecan-1 expression is required to maintain tumor-initiating cells (Shimada et al. 2013). One of the CSC features is resistance to chemotherapy (Vitale et al. 2019). High preoperative Syndecan-1 serum levels were significantly associated with poor disease-free survival and contribute to the resistance to various chemotherapeutic drugs in colorectal cancer patients. Mechanistically, the chemotherapy resistance can be linked to Syndecan-1 shedding, which induces the EGFR signaling pathway (Wang et al. 2014). It seems that a similar mechanism of chemotherapy resistance and tumor relapse does exist in castration-resistant prostate cancer, pancreatic cancer cells, and multiple myeloma, involving the shedding of Syndecan-1 (Szarvas et al. 2018; Ramani and Sanderson 2014). Therefore, the presence of shed Syndecan-1 ectodomain in serum could act as a predictive marker for chemotherapy response and possible recurrence for some tumors.

Apart from its regulatory role in the stemness of solid tumors, Syndecan-1 has a crucial role in soft tissue tumors, namely adipose tissue tumors. Syndecan-1 is highly overexpressed in dedifferentiated liposarcoma in comparison to normal adipose

tissue and lipomas (Zaragosi et al. 2015). Syndecan-1 expression favors the proliferation of undifferentiated adipocyte progenitors and impairs their adipogenic differentiation; culminating in stabilizing the undifferentiated phenotype (Zaragosi et al. 2015).

Biological and molecular functions of Syndecan-1 (CD138) have been extensively studied in hematological tumors, especially multiple myeloma. Syndecan-1 (CD138) is frequently expressed on the surface of normal and malignant plasma cells, which is known as multiple myeloma. In multiple myeloma, the CD138-positive mature cancer cells display proliferative activity and chemosensitivity with developmental plasticity that could be dedifferentiated back into the CD138-negative chemoresistant progenitor quiescent pre-plasma cell with increased ALDH1 activity, whereby this plasticity is governed by macrophage inhibitory factor-1 (MIF-1) (Joseph et al. 2018; Reghunathan et al. 2013; Matsui et al. 2008). In addition, compared to CD138-positive multiple myeloma, an increased STAT3 and ERK1/2 activation were observed in CD138-negative cells, which displayed low response to chemotherapy. The presence of that chemoresistant CD138-negative subpopulation within the bone marrow is the main driving force of tumor relapse in multiple myeloma patients (Fuhler et al. 2010). It was reported that putative myeloma stem cells can be eliminated by CD138-specific CAR-T cells without off-target cytotoxicity against normal epithelial or endothelial cells (Sun et al. 2019b). Further, bone marrow CD138-positive multiple myeloma cells are the main source of hedgehog (Hh) ligand sonic hedgehog (SHH), the key player implicated in malignancy of multiple myeloma (Liu et al. 2014). Autocrine SHH augments CD138-positive myeloma cell proliferation and spontaneous and stress-induced apoptosis (Liu et al. 2014). The clonogenic SP cells with stem-like features express CD138 antigen relative to non-SP cells in multiple myeloma cell lines (Jakubikova et al. 2011). CD138 expression is a relevant marker for tumor cell isolation since rare circulating plasma cancer cells in the blood of multiple myeloma can be captured by a microfluidic cell capture via CD138 antigen expression for the subsequent fluorescence-based assays (Qasaimeh et al. 2017). This is in contrast to another study, whereby the authors demonstrated an alternative biomarker set to detect drug-resistant minimal residual and circulating multiple myeloma cells independent of CD138 expression because of its reduced expression following bortezomib treatment-induced hypoxia (Muz et al. 2016). Of note, treatment of hypoxic multiple myeloma cell lines KMS-12BM and RPMI 8226 with the differentiation inducer all-trans retinoic acid (ATRA) restored CD138 expression, repressed expression of stem cell-specific transcription factor, and sensitized multiple myeloma cells to bortezomib under hypoxia (Kawano et al. 2013). In another study, data showed no differences between CD138^{high} and CD138^{low} subpopulations in 8 multiple myeloma cell lines based on phenotypic, genomic, and functional profiling, and that both subsets have a comparable sensitivity to the anticancer drugs bortezomib, melphalan, and doxorubicin (Paino et al. 2014). Moreover, both CD138^{high} and CD138^{low} cells had self-renewal capacity when engrafted in CB17-SCID mice (Paíno et al. 2014). In this context, CD138-positive human multiple myeloma plasma cells have the same potential to propagate multiple myeloma clones similar to CD138-negative cells, but

at a slower rate when engrafted in the SCID-rab model (Hosen et al. 2012). Syndecan-1 is not a static cell membrane molecule, but it can be cleaved from the cell membrane to generate a soluble form via protease-mediated shedding (Piperigkou et al. 2016; Wang et al. 2005). Therefore, the discrepancy for the role of Syndecan-1 in multiple myeloma could be partially attributed to: the role of shedding form of Syndecan-1, a process mediated by HS-degrading heparanase in the tumor microenvironment of multiple myeloma (Mahtouk et al. 2007; Teixeira and Götte 2020), the difference between the multiple myeloma cells used in different studies (e.g., cell lines vs. primary cells), methods of subpopulation isolation, and the distinctive biology of the disease among patients with different clinical stages of cancer progression (Ghosh and Matsui 2009) (Fig. 12.3).

12.4 Conclusions and Perspective

CSCs are a subpopulation of cells within a tumor that are of particular translational relevance, as they have been linked to recurrence after an otherwise successful therapy, due to their property of increased resistance to chemo- and radiotherapy (Greve et al. 2012; Vitale et al. 2019). The function of CSCs depends on multiple signaling pathways, which are triggered by ligands of the CSC niche or cell-autonomous stimulation via hyperactivation within the tumor cell due to constitutively activating mutations in signaling receptors, gene duplications in pathway constituents or promotor mutations driving the transcription of stimulating ligands and receptors or downregulating their repressors (Vitale et al. 2019). Notably, key stemness-associated pathways dysregulated in cancer are modulated by (cell surface) heparan sulfate, including Wnt-, hedgehog-, Notch-, FGF-, and NF-kB/IL-6/STAT3-signaling (Vitale et al. 2019; Espinoza-Sánchez and Götte 2020; Jones et al. 2021; Rai and Wang 2021). Among the core proteins carrying heparan sulfate chains, recent results demonstrate important roles for Syndecan-1 as a modulator of CSC function. The expression of Syndecan-1 is not only dysregulated in numerous tumor entities, but is also of relevance for signaling through the aforementioned pathways, serving as multifunctional integrator of stemness-related signaling (Karamanos et al. 2018; Vitale et al. 2019). Consequently, attenuation of Syndecan-1 expression has been linked to alterations in the CSC phenotype, for example, to a reduction of mammosphere formation and reduced CSC marker expression in the case of (inflammatory) breast cancer (Ibrahim et al. 2013, 2017).

How can this knowledge be exploited? Apart from using Syndecan-1 and its associated molecular pathways as diagnostic and prognostic markers (Ibrahim et al. 2017; Götte and Kovalszky 2018; Espinoza-Sánchez et al. 2019; Teixeira and Götte 2020), pharmacological interference with Syndecan-1 function may provide a means of targeting the CSC population. Indeed, drugs targeting several stemness-associated signaling pathways are currently under evaluation in clinical trials, including several phase I trials for Wnt pathway inhibitors, and several phase II trials for Notch and

hedgehog pathways inhibitors, respectively (Clara et al. 2020). A targeting of Syndecan-1 will expand the spectrum of available therapies and may provide an advantage due to the pleiotropic involvement of Syndecan-1 in multiple stemness-associated pathways (Fig. 12.4). A direct approach is the use of antibodies directed against Syndecan-1. For example, the application of monoclonal antibody OC-46F2 blocked both tumor growth and blood vessel maturation in experimental models of ovarian cancer and melanoma (Orecchia et al. 2013). Moreover, the monoclonal anti-Syndecan-1 antibody B-B4 has been successfully used as an antibody–drug conjugate to target multiple myeloma cells in different preclinical studies, leading to tumor cell death in vitro and in vivo, and to an increased antitumoral response by NK cells (Tassone et al. 2004; von Strandmann et al. 2006; Ikeda et al. 2009). Syndecan-1 has also been successfully used as a target for CAR-T cells in preclinical studies on multiple myeloma (Espinoza-Sánchez and Götte 2020). In addition, the B-B4 antibody has been successfully employed for in vivo imaging and radioimmunotherapy of breast cancer xenografts (Rousseau et al. 2011). A potential caveat associated with the preclinical animal studies is, however, that the human-specific Syndecan-1 antibodies will not react with the murine host Syndecan-1, resulting in a more favorable side effects profile than can be expected in humans. Another means for modulating Syndecan-1 expression is the application of microRNA drugs, which are currently under evaluation in clinical trials by several pharmaceutical companies (Rupaimoole and Slack 2017). For example, therapeutics based on the microRNAs miR-10b, miR-122-5p, miR-145, miR-200b, and miR-494, known to target Syndecan-1 directly, or modulate its expression in an indirect manner, have shown promising results in several preclinical disease models and await clinical translation (Ibrahim et al. 2012, Schneider et al. 2013, Asuthkar et al. 2014; Fujii et al. 2015; Piperigkou et al. 2017; Rupaimoole and Slack 2017; Uen et al. 2018, Piperigkou et al. 2020). Apart from these direct approaches, an indirect modulation of Syndecan-1 expression and function can be utilized for targeting Syndecan-1-dependent pathways. For example, it has been shown that the widely used bisphosphonate zelodronate downregulates Syndecan-1 in breast cancer cell lines in vitro, resulting in an inhibition of adhesion, migration, and invasive properties (Dedes et al. 2012). Moreover, the Syndecan-1 function is modulated by ectodomain shedding, as demonstrated, for example, in MCF-7 breast cancer cells, which showed a more aggressive and invasive phenotype upon expression of soluble compared to membrane-bound Syndecan-1 (Nikolova et al. 2009). As Syndecan-1 can be cleaved from the membrane in a gamma-secretase-dependent manner (Pasqualon et al. 2015), the use of gamma-secretase inhibitors may be a means of simultaneously targeting the Syndecan-1 and Notch signaling pathways in CSCs (Ibrahim et al. 2017; Ramirez Williams et al. 2019; Clara et al. 2020). Finally, in cancer and inflammation, Syndecan-1 is functionally closely linked to the heparan sulfate degrading enzyme heparanase (Teixeira and Götte 2020). In myeloma, the shedding of Syndecan-1 is linked to heparanase and associated with endothelial invasion and angiogenesis (Purushothaman et al. 2010). Therefore, the use of heparanase inhibitors that have already entered clinical studies may be a means of indirectly regulating Syndecan-1 function in CSCs, while targeting invasive cancer

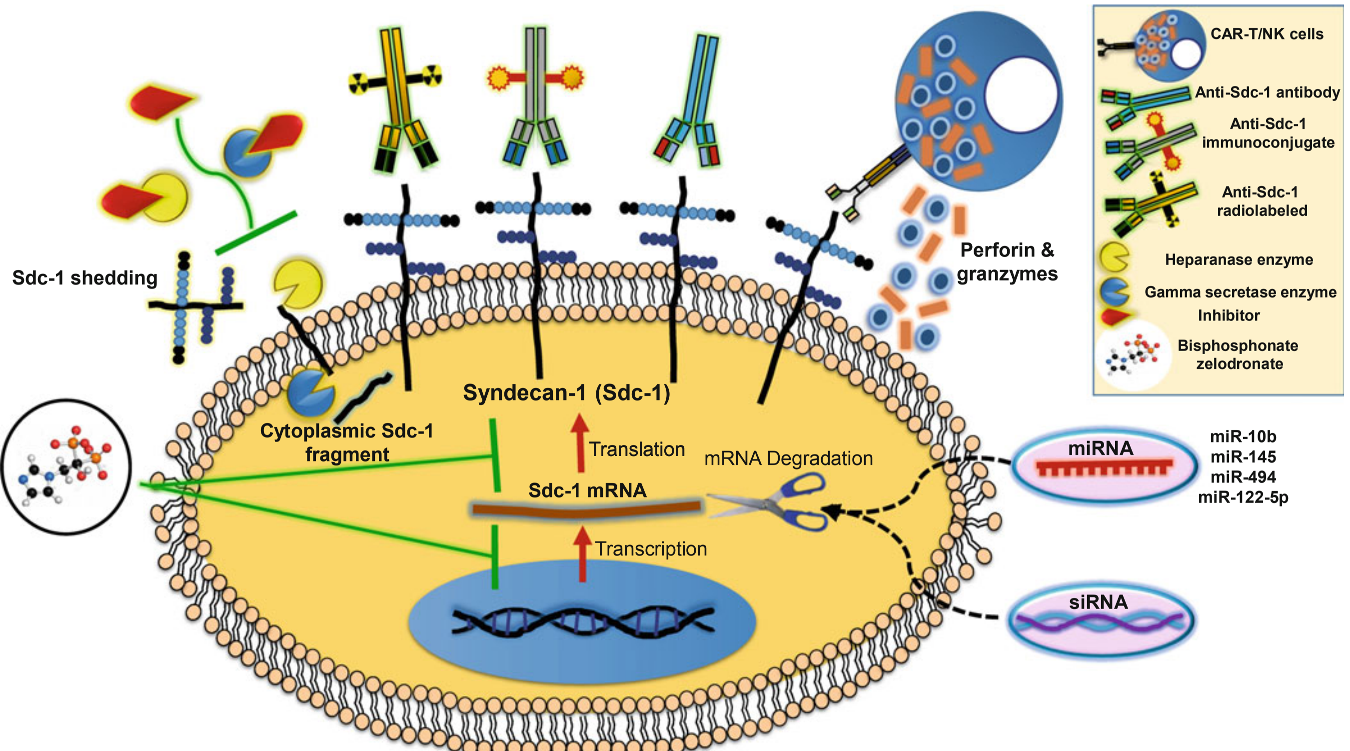

Fig. 12.4 A Schematic diagram for promising therapeutic strategies that can be used for Syndecan-1 targeting in cancer. The extracellular domain of Syndecan-1 can be targeted by anti-syndecan-1 antibodies, immunoconjugates linked to cytotoxic drugs or receptor-specific ligands on natural killer cells to induce their activation, radiolabeled antibodies (immunoradiotherapy), or CAR-T/NK cells. Syndecan-1 can be also targeted employing siRNAs or miRNA mimetics that induce mRNA degradation. The pharmaceutical agent bisphosphonate zelodronate interferes with Syndecan-1 expression at both mRNA and protein levels. Moreover, application of pharmacological inhibitors against heparanase-induced extracellular domain shedding of Syndecan-1 or cleaving of its intracellular domain by gamma-secretase can serve as further therapeutic perspectives

cell behavior via heparanase inhibition (Weissmann et al. 2016; Noseda and Barbieri 2020; Giannini et al. 2020). Similarly, heparinoids and heparan sulfate mimetics could interfere with stemness-related functions of the Syndecan-1 heparan sulfate chain and may further widen the spectrum of drugs targeting Syndecan-1 function (Yip et al. 2006; Cassinelli et al. 2020; Chhabra and Ferro 2020), however, these drugs have not been evaluated in a CSC context so far. Overall, the encouraging results in preclinical models provide a positive perspective for the translation into clinical trials in the not too distant future.

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