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Heparan sulfate: a key regulator of embryonic stem cell fate

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Abstract

Heparan sulfate (HS) belongs to a class of glycosaminoglycans and is a highly sulfated, linear polysaccharide. HS biosynthesis and modification involves numerous enzymes. HS exists as part of glycoproteins named HS proteoglycans, which are expressed abundantly on the cell surface and in the extracellular matrix. HS interacts with numerous proteins, including growth factors, morphogens, and adhesion molecules, and thereby regulates important developmental processes in invertebrates and vertebrates. Embryonic stem cells (ESCs) are distinguished by their characteristics of self-renewal and pluripotency. Self-renewal allows ESCs to proliferate indefinitely in their undifferentiated state, whereas pluripotency implies their capacity to differentiate into the three germ layers and ultimately all cell types of the adult body. Both traits are tightly regulated by numerous cell signaling pathways. Recent studies have highlighted the importance of HS in the modulation of ESC functions, specifically their lineage fate. Here, we review the current advances that have been made in understanding the structural changes of HS during ESC differentiation and in deciphering the molecular mechanisms by which HS modulates cell fate. Finally, we discuss the applications of heparinoids and chemical inhibitors of HS biosynthesis for the manipulation of ESC culture and directed differentiation.

Keywords

cell fate; embryonic stem cell; heparan sulfate; signaling

Introduction

Embryonic stem cells (ESCs) are derived from the inner cell mass (ICM) of the preimplantation blastocyst. They are unique in their potential to differentiate into all cell types representative of the three germ layers of the embryo, a property that is defined as pluripotency (Evans and Kaufman, 1981; Martin, 1981). ESCs retain pluripotency through a process of self-renewal, which allows ESCs to proliferate infinitely as undifferentiated entities. These properties make ESCs a unique system to study early embryonic

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development and cell fate decisions, and endow ESCs with great promise for the development of cell replacement therapies for degenerative disease and injury.

ESCs were first isolated from the mouse ICM in 1981 and were later derived from the human blastocyst in 1998 (Martin, 1981; Thomson et al., 1998). Since ESCs became available, substantial effort has been invested into understanding the mechanisms that regulate their self-renewal and differentiation. Numerous studies have revealed a comprehensive list of factors that control ESC self-renewal, including growth factors, cytokines, transcription factors, epigenetic modifiers, and non-coding RNAs. When allowed to differentiate in serum-containing culture medium, ESCs will spontaneously develop into a heterogeneous cell population with multiple cell types. Spontaneous differentiation may occur in adherent culture or suspension culture where colonies called embryoid bodies (EBs) form. These *in vitro* differentiation systems represent valid models of early embryonic development, as molecular and cellular events associated with early lineage establishment are closely recapitulated (Loebel et al., 2003). As more homogenous cell populations are desired, differentiation can be directed by replacement of fetal calf serum with combinations of selected growth factors.

In metazoans, the formation of the complex body pattern during development is controlled by secreted signaling molecules, including members of the FGF, TGF- β , and Wnt/Wingless families. They participate in key developmental functions such as differentiation, migration, and proliferation. The aforementioned growth factors also direct cell fate decisions of ESCs into cell types representative of the three germ layers and their derivatives. As such, cocktails of developmental growth factors are used to direct ESC differentiation into cell types such as cardiac progenitors and neural precursor cells (Keller, 2005; Wobus and Boheler, 2005). Despite recent efforts and advancements, a major challenge in the field remains to achieve stable and homogenous differentiation of ESCs into cell types that may eventually be usable for curative purposes. New insights into the complexities of cell signaling and the molecular mechanisms that modulate cell fate are necessary to improve the efficacy of ESC differentiation.

Heparan sulfate (HS) is a linear, highly sulfated polysaccharide and is present on the cell surface and in the extracellular matrix (ECM) of virtually all mammalian tissues (Bernfield et al., 1999). Proteins that interact with HS include numerous developmentally important signaling molecules, growth factors, and ECM components. HS chains regulate developmental signaling by acting as co-factors through a variety of mechanisms that include but are not limited to maintenance of morphogen gradients and co-receptor functions (Lin, 2004; Kreuger et al., 2006; Bishop et al., 2007). Beyond development, HS modulates various other physiological functions in mammals that are discussed elsewhere (Bishop et al., 2007; Fuster and Wang, 2010; Sarrazin et al., 2011). HS is abundantly expressed in ESCs and becomes further modified in a cell type-specific manner as ESCs undergo differentiation (Johnson et al., 2007; Nairn et al., 2007). Given the critical importance of HS in the modulation of growth factors, cytokines, and matrix biology during vertebrate development, it is not surprising that HS represents an important regulator of stem cell fate. Recent efforts have used various chemical and genetic approaches to address the function and structure-function relation of HS in stem cell self-renewal and differentiation. Confounded by the various strategies of HS manipulation, conclusions and interpretations from numerous studies have not been straightforward. In this review, we aim to reconcile data from recent studies in order to provide a clearer picture of HS and its function in stem cell self-renewal and differentiation with a major focus on ESCs.

HS biochemistry

HS is a linear polysaccharide and belongs to the family of glycosaminoglycans (GAG). HS is composed of glucuronic acid (GlcA) and iduronic acid (IdoA) residues as well as 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 that initiate, extend, and modify the HS chain, giving rise to a highly heterogeneous structure of mature HS (Figure 1). The biosynthesis of HS takes place primarily in the Golgi apparatus and all enzymes involved, except H3ST-1, are typical type II transmembrane proteins (Sugahara and Kitagawa, 2002). The biosynthesis of HS is initiated by the addition of a GlcNAc residue to the GlcA β 1-3Gal β 1-3Gal β 1-4Xyl β 1-O-tetrasaccharide that is linked covalently to HS proteoglycan (HSPG) protein cores. Chain elongation of HS is carried out by members of the EXT gene family, which alternately add GlcNAc and GlcA residues. EXT1 and EXT2 are co-polymerases that possess both GlcNAc and GlcA transferase activity and are the main enzymes involved in the extension of the HS chain. Both EXT1 and EXT2, when expressed individually, show little polymerizing activity. Significant polymerizing activity is achieved only when EXT1 and EXT2 are complexed together in the Golgi apparatus (McCormick et al., 1998, 2000). Other members of the EXT family include EXT-like (EXTL) 1–3 and are believed to assist in the initiation and elongation of the HS chain. However, their exact functions are still poorly understood (Kitagawa et al., 1999; Kim et al., 2001). Following chain elongation, N-deacetylase/N-sulfotransferases (Ndsts) act on discrete regions of the HS precursor, replacing N-acetyl groups with N-sulfates. There are four Ndst isoforms (Ndst1–4), which differ in the ratios of their relative enzyme activities of N-deacetylation to N-sulfation (Aikawa et al., 2001). Regions of N-sulfation then act as substrates for a variety of enzymes that catalyze additional modifications, including epimerization of GlcA to IdoA by C5-epimerase followed by 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 C5-epimerase and HS2st have been found (Sugahara and Kitagawa, 2002). Sulfation and epimerization modifications are not evenly spread but occur in clusters, resulting in regions rich in N-sulfated and epimerized residues (S-domains) and mostly unmodified residues (NS-domains). In this way, a vast number of highly variable HS sequences are generated. Intriguingly, the polymer-modifying reactions are not random but are instead tightly regulated in a context-specific manner, making HS chains subject to alterations during development and ageing. It is generally accepted that the sequence and sulfation patterns of the HS chain confer specificity to its interactions with its binding partners. Such complexity allows HS not only to interact with a plethora of molecules and compounds but also to exhibit spatiotemporal regulatory functions during development (Esko and Selleck, 2002; Rapraeger, 2002; Bishop et al., 2007).

During biosynthesis, HS chains are covalently attached to core proteins to form HSPGs. Dictated by their core proteins, HSPGs are expressed either on the cell surface, in the ECM, or in secretory granules where the HS moieties mediate the majority of the biological functions of HSPGs by interacting with growth factors, growth factor-binding proteins, extracellular proteases, protease inhibitors, chemokines, morphogens, and adhesive proteins (Bernfield et al., 1999; Sarrazin et al., 2011). There are three major classes of HSPGs: transmembrane syndecans, glycosylphosphatidylinositol-anchored glypicans, and extracellular HSPGs such as perlecan. Additional HS-containing proteoglycans include CD44 and betaglycan that are membrane-spanning ‘part-time’ cell surface proteins. Syndecans, glypicans, and perlecan carry HS chains as a constant feature, whereas ‘part-time’ proteoglycans carry HS only in some instances. Most cell types express multiple

syndecans and glypicans, and their expression is typically cell type- and developmental stage specific (Bernfield et al., 1999).

Alterations of HS structure upon stem cell fate commitment

HS biosynthetic enzyme expression is developmentally orchestrated and different isozymes of the enzyme family members are expressed in a spatiotemporal fashion. As a consequence, HS chains are continuously remodeled as stem and progenitor cells progress through various lineage stages. Direct analysis of HS structure and content alongside expression assays of HS biosynthetic genes in undifferentiated and differentiated ESCs have led to a better understanding of developmental HS remodeling. The purification of HS from ESCs that are subjected to various differentiation regimens reveals qualitative as well as quantitative changes of HS upon cell fate commitment. Around 80% of GAGs produced in ESCs is HS, with a lesser amount produced as chondroitin sulfate (CS) (Lin et al., 2000; Nairn et al., 2007). Irrespective of lineage fate, general trends such as an increase in overall HS content and sulfation along differentiation have been observed. Interestingly, the increase in overall HS content is accompanied by only small increases in transcript levels of genes of the *EXT* family and suggests that other mechanisms of enzyme regulation account for the increase in HS content. Apart from increases in HS content, it is striking that the production of other GAGs, including hyaluronic acid and CS/dermatan sulfate (DS), becomes induced during ESC differentiation and is paralleled by increases in the majority of HS as well as CS/DS core proteins (Nairn et al., 2007). ECM components become increasingly expressed during EB formation and are found in microenvironments of EBs (Shukla et al., 2010). Hence, increased GAG production during differentiation may accommodate an increase in ECM deposition.

HS composition analyses of undifferentiated ESCs revealed that their HS chains carry surprisingly little sulfate, with only around 30% of N-sulfation. The possible function of this low-sulfated form of HS is unclear, but may serve to shield ESCs from differentiation-induction signaling, as will be discussed in the next section of this review. As ESCs transition into committed cell types, their HS chains become increasingly sulfated (Johnson et al., 2007; Nairn et al., 2007; Hirano et al., 2012). Changes in sulfation upon lineage commitment arise primarily as a result of increased N-, 3-O-, and 6-O-sulfation. Upregulation of sulfotransferase mRNAs is lineage specific and appears to be a major contributor for increased sulfation. For instance, transition from undifferentiated ESCs into a Sox1⁺ neural population is accompanied by strong increases in Ndst-4, 3-HSst-3a, and 3-HSst-5, which are predominantly expressed in fetal brain tissue (Mochizuki et al., 2003; Yabe et al., 2005; Johnson et al., 2007). In contrast, enrichment of extraembryonic cell types is paralleled by increases in Ndst1, Ndst2, HS6st2, and Hs3sta1 (Nairn et al., 2007). Such lineage-specific expression of HS biosynthetic enzymes will give rise to cell type-specific HS that displays selectivity toward growth factors and will directly affect the cell's differentiation potential, as has been shown from both hematopoietic and neural differentiation systems. Hematopoietic differentiation of ESCs can be achieved through formation of EBs. During EB differentiation, transient populations of *Brachyury*-positive mesodermal cells will give rise to endothelial progeny in response to VEGF. Using HS-specific phage-display antibodies, a specific HS4C3 epitope that requires N- and 6-O-sulfate and a single critical 3-O-sulfate for high-affinity binding is identified in mesodermal subpopulations. The HS4C3⁺ subpopulation displays enhanced hematopoietic potential compared with HS4C3⁻ mesoderm cells, an indication that this epitope is highly functional in the context of hematopoietic development (Baldwin et al., 2008).

Differences in differentiation potentials among cells arise from the remodeling of HS structures and will ultimately be the result of changes in the affinity of HS for specific cell

signaling molecules and will allow for selective cell fate decisions. For example, the HS of neural progenitor cells (NPCs) shows a decrease in FGF2 binding compared with binding to HS of ESCs and is concomitant with Sox1 expression (Johnson et al., 2007). The differentiation step of ESCs into NPCs is accompanied by increases in N- and 6-O-sulfation and, to a lesser extent, 2-O-sulfation. Subsequently, as neuroepithelial precursors switch from proliferation to neuronal differentiation, their HS will undergo changes in the pattern of 6-O-sulfation as well as total HS chain length. This switch coincides with a switch from potentiation of FGF2 to FGF1 signaling, which is needed for differentiation of NPCs into terminally differentiated neural cell types (Brickman et al., 1998). Hence, it appears that HS structure changes facilitate a sequential change in affinity for FGF isoforms. During the formation of NPCs, the remodeling of HS structures decreases the affinity for FGF2, which may prime NPCs for differentiation by allowing increases in affinity for other FGFs such as FGF1. Gradually, a switch in affinity from FGF2 to FGF1 will ensue and ultimately drive neural differentiation. *In situ* binding assays with embryonic tissues have revealed an additional layer of specificity in HS affinity to FGFs. Even different FGF-FGFR complex combinations display differential affinity toward developmental HS motifs and emphasize the critical role of HS-FGF-FGFR ternary complexes in regulating embryogenesis (Allen and Rapraeger, 2003).

In summary, several studies have demonstrated that HS is remodeled during ESC differentiation and that structure alterations appear to drive lineage choices. ESCs provide an attractive *in vitro* system from which cells of specific developmental stages can be relatively easily enriched, propagated, and analyzed for their GAG content and structure. Stage-specific reporter cell lines that allow for convenient cell sorting combined with differentiation protocols that direct lineage fate choices are ideal tools to study HS structure-function relations that pertain during vertebrate development and will undoubtedly advance our understanding of complex HS structure changes and its modulation of development as well as ESC differentiation.

HS is required for lineage commitment of ESCs

The role of HS in ESC self-renewal has been studied in recent years using various ES cell lines that are either deficient in HS or carry undersulfated HS. These include EXT1 null (EXT1^{-/-}), EXT1^{cn/cn} [a mouse ESC (mESC) line generated through the ablation of a conditional EXT1 allele *in vitro*], Ndst1/2^{-/-}, stably transfected siRNA-EXT1, and transiently transfected si-EXT1 (EXT1-KD) ESCs. Self-renewal phenotypes differ markedly between HS mutant lines. EXT1^{-/-}, EXT1^{cn/cn}, Ndst1/2^{-/-}, and stable siRNA EXT1 ESCs all retain their self-renewal capacity over extended culture periods (Holmborn et al., 2004; Johnson et al., 2007; Kraushaar et al., 2010; Lanner et al., 2010; Pickford et al., 2011). In contrast, transiently transfected siRNA-EXT1-KD ESCs display compromised self-renewal (Sasaki et al., 2008). The discrepancies between cell lines may be caused by differences in the efficiency of HS depletion. The siRNA-EXT1-KD ESCs retain around 20% of residual HS, which exhibits changed sulfation patterns compared with the HS of wild-type ESCs (Sasaki et al., 2008). Therefore, residual HS of the siRNA-EXT1-KD ESCs may affect pro- and antidifferentiation signals differently than the complete lack of HS in EXT1^{-/-} and EXT1^{cn/cn} ESCs. However, transient knockdown of EXT1 may reveal an immediate effect that results in compromised self-renewal more readily than stable cell lines that are subjected to selection over several passages. Yet, the majority of studies to date have demonstrated that endogenous HS is dispensable for self-renewal but becomes critical when ESCs are challenged to differentiate upon leukemia inhibitory factor (LIF) withdrawal. Spontaneous adherent differentiation, EB suspension culture, and serum-free differentiation into neural precursors have demonstrated that complete loss of HS or undersulfation of HS leads to failure of the lineage commitment. HS mutant ESCs, including EXT1^{-/-},

EXT1^{cn/cn}, and Ndst1/2^{-/-} ESCs, fail to differentiate into cell types representative of ecto-, meso-, and endoderm but still retain the expression of ESC markers (Johnson et al., 2007; Kraushaar et al., 2010; Lanner et al., 2010; Holley et al., 2011; Forsberg et al., 2012).

Several HS-binding signaling molecules have been shown to critically regulate mESC self-renewal, including BMP, Wnt, and FGFs. BMP4 has antidifferentiation effects primarily by activating the transcription factor Id1, which in turn is required for maintaining high Nanog levels during self-renewal (Ying et al., 2003). Wnt signaling converges with the LIF/STAT3 pathway by upregulating STAT3 expression and thereby contributes toward maintenance of pluripotency (Hao et al., 2006). Additional integration of Wnt signaling into the self-renewal circuit is achieved through activation of the *T-cell factor 3* (*Tcf3*), which co-occupies several promoters in association with additional pluripotency factors, including Nanog and Oct-4 (Cole et al., 2008). In contrast, FGF signaling and activation of its downstream mediator MAP kinase are essential requirements for lineage commitment, as has been shown from studies of ESCs treated with FGFR- and MAPK inhibitors (Hamazaki et al., 2006; Kunath et al., 2007; Ying et al., 2008). Closer examination of signaling pathways that are involved in ESC self-renewal showed that HS is required for normal cell surface FGF binding and subsequent intracellular signaling, which is in line with a role of HS as a co-receptor in formation of the ternary HS-FGF-FGFR signaling complex. Therefore, an important role of HS is to promote exit from ESC self-renewal by facilitating FGF signaling. In addition to their failure to downregulate pluripotency genes, including *Nanog*, upon removal of LIF, EXT1^{cn/cn} ESCs also express higher levels of *Nanog* in their undifferentiated state in the presence of LIF (Kraushaar et al., 2010). Therefore, loss of HS also results in a more naïve ground state in ESCs and shows further that HS functions to tune ESCs to be in a less naïve state.

Although perhaps counterintuitive at first, this role of HS in controlling the cell fate of ESCs may best be explained in the context of FGF signaling in the early embryo. The ICM of the blastocyst exhibits heterogeneity, as reflected by the expression of Nanog and the primitive endoderm (PE) marker GATA6 in a non-overlapping fashion (Yamanaka et al., 2010). The Nanog⁺ and GATA6⁺ subpopulations of the ICM will give rise to epiblast and PE, respectively. ESCs mimic cells of the ICM in this way under serum-containing conditions in the presence of LIF. Similar heterogeneity is found in ESC cultures where the two lineage markers are expressed in a non-uniform manner. Generally speaking, two subpopulations of ESCs exist; one displays an expression profile of Nanog⁺Gata6⁻ epiblast-like cells, which are primed to differentiate into cell types of all germ layers. The other subpopulation is Nanog⁻Gata6⁺ and solely differentiates into extraembryonic endodermal cell types (Singh et al., 2007). When either subpopulation is isolated, the original equilibrium between Nanog⁺ and Nanog⁻ ESCs is reinstated, suggesting the existence of a metastable ESC state in which cells are not predetermined to take on either cell fate but rather have the capacity to spontaneously and reversibly take on alternative cell fates. FGF4 is the predominant isoform of FGF that is expressed in the 8–16 cell morula and becomes gradually restricted to epiblast cells of the ICM (Yuan et al., 1995). *In vivo* and *ex vivo* experiments have shown that the segregation of epiblast and PE is dependent on FGF4 signaling (Yamanaka et al., 2010). Also, treatment of ESCs with FGFR inhibitors leads to less heterogeneity and a larger percentage of Nanog⁺GATA6⁻ cells, showing that inhibition of FGF signaling results in the failure to establish a PE population (Hamazaki et al., 2006). As learned from EXT1^{cn/cn} and Ndst1/2^{-/-} ESCs, HS and its N-sulfation are critical for FGF signaling (Kraushaar et al., 2010; Lanner et al., 2010). In line with the proposed role of FGF signaling and its induction of PE-like cells, EXT1^{cn/cn} ESCs express elevated levels of Nanog mRNA in their undifferentiated state and Ndst1/2^{-/-} cells exhibit greater homogeneity among ESCs with a more uniform number of Nanog⁺ cells. Furthermore, treatment of blastocysts with NaClO₃, a sulfation inhibitor, prevents PE formation, further demonstrating that HS is involved in the

lineage specification between epiblast and PE (Lanner et al., 2010). Hence, overall, HS facilitates FGF signaling and induces the MAPK activity required for Nanog downregulation and PE formation within a metastable ESC population. A 'low-sulfated' HS species may shield ESCs from excessive FGF signaling that would otherwise result in premature loss of 'stemness' and pluripotency. By means of Nanog downregulation, HS further facilitates germ layer differentiation of epiblast cells, as suggested from EXT1^{cn/cn} ESCs that fail to differentiate into ecto-, meso-, and endodermal cell types in the absence of LIF (Kraushaar et al., 2010). In summary, HS appears to be required for an initial epistable ESC cell state and segregation of epiblast and PE, and then, subsequently for germ layer differentiation (Figure 2A).

Examination of additional signaling pathways involved in ESC self-renewal revealed defects in BMP4 signaling in EXT1^{-/-} ESCs (Holley et al., 2011; Kraushaar et al., 2012). Yet, EXT1^{-/-} ESCs display enhanced rather than compromised self-renewal despite lower than normal phospho-SMAD and ID1 levels. The observed phenotype may be explained by the relative contributions of BMP4 and FGF signaling toward the maintenance of self-renewal. Studies have demonstrated that ESCs can be maintained in a 'naïve ground state' in the absence of extrinsic signals such as BMP4 as long as MAPK activity is suppressed with chemical inhibitors (Ying et al., 2008). Hence, inhibition of MAPK activity is considered elementary to self-renewal and does not need BMP4 activity. In fact, when BMP4 signaling is active, a major mechanism of promoting self-renewal is its ability to inhibit ERK signaling (Qi et al., 2004). The instructive role of ERK inhibition for self-renewal may explain why EXT1^{-/-} and EXT1^{cn/cn} ESCs display an enhanced self-renewal phenotype as a result of the dominant effect mediated by the inhibition of FGF signaling and the associated net effect of MAPK levels irrespective of impaired BMP4 activity. In summary, HS carries out dual roles in self-renewal through two separate pathways: it promotes as well as inhibits self-renewal through modulation of BMP4 and FGF signaling, respectively. However, the regulatory role on FGF signaling appears to be dominant.

The role of HS in Wnt signaling, another important pathway involved in ESC self-renewal, is less clear, as some studies report HS as a negative regulator and some as a positive regulator of Wnt signaling. An increase in Wnt signaling activity was observed in EXT1^{cn/cn} ESCs as opposed to a reduction in Wnt activity that was reported from a knockdown study of EXT1 (Sasaki et al., 2008; Kraushaar et al., 2012). At least in the context of glypican 4, an abundant glypican expressed by ESCs, HS may be a positive regulator of self-renewal, although it has not been demonstrated whether HS chains or core proteins are the actual mediators of Wnt signaling (Fico et al., 2012). Recently, Fas signaling was reported to be implicated in promoting ESC self-renewal and was shown to depend on 3-O-sulfation of HS for the activation of this pathway (Hirano et al., 2012). Although the role of Fas signaling in ESC self-renewal needs further characterization, this and other studies have merged the strict structure-function relations of HS into the modulation of ESC self-renewal.

HS modulates ESC pluripotency

Embryonic lineage fates along the three germ layers and subsequent differentiation steps are regulated by numerous growth factors and morphogens. Many of them contain heparin-binding domains and may be modulated by HS. The increase in HS sulfation and the cell type-specific appearance of select HS motifs during various stages of differentiation indicate that lineage specification may be dependent on HS. This possibility has been explored with *Ndst1*^{2-/-} and *EXT1*^{-/-} ESCs. These studies show that HS is required initially to induce gene expression associated with the three germ layers and also to differentiate into mid- and terminally differentiated cell types such as endothelial cells, osteoblasts, adipocytes, hemangioblast-type cells, and neurons (Jakobsson et al., 2006; Johnson et al., 2007; Lanner

et al., 2010; Holley et al., 2011; Forsberg et al., 2012). Considering the retention of pluripotency gene expression and alkaline phosphatase activity that were reported from cell populations examined in these studies, it appears likely that the failure of *EXT1*^{-/-} and *Ndst1*^{1/2}^{-/-} ESCs to differentiate into the aforementioned cell types may be attributable to the block in initial cell fate commitment. We recently applied a modified adherent cell differentiation system that overcomes the *EXT1*^{cn/cn} cell commitment block through addition of high doses of FGF2 to culture medium during the initial and critical phase of self-renewal exit. The most striking hallmarks of aberrant *EXT1*^{cn/cn} differentiation into germ layers were their failure to induce the pan-mesoderm marker Brachyury and abnormal expression of genes involved in dorso-ventral patterning, revealing the critical importance of HS in ESC differentiation into mesoderm (Kraushaar et al., 2012).

Several developmental signaling pathways regulate mesoderm differentiation in ESCs as well as in early embryonic development, including FGF/MAPK, BMP4, and Wnt (Lindsley et al., 2006; Lengerke et al., 2008; Willems and Leyns, 2008; Hansson et al., 2009). BMP signaling has well-defined roles in the dorso-ventral patterning of mesoderm during gastrulation and in ESCs. BMP4 signaling induces ventral-posterior mesoderm and inhibits anterior mesoderm, the latter of which will give rise to definitive endoderm (Hemmati-Brivanlou and Thomsen, 1995). In addition, neuroectodermal differentiation is potentially inhibited by BMP4 (Wilson and Hemmati-Brivanlou, 1995; Finley et al., 1999; Kawasaki et al., 2000). In line with the roles of BMP4, *EXT1*^{cn/cn} ESCs display reduced expression of posterior mesoderm genes such as *Evx1* and *Mesp1* and overexpression of endodermal genes such as *Sox17* and *Foxa2*. Restoration of BMP signaling rescues aberrant mesoderm differentiation and shows that HS facilitates BMP signaling to mediate mesoderm differentiation (Kraushaar et al., 2012). Chlorate treatment, which inhibits the sulfation of HS chains, also results in defects of BMP4 signaling and induces accelerated neural differentiation at the expense of mesodermal differentiation (Sasaki et al., 2010). Such enhanced neural differentiation was also seen in PAPST1 and PAPST2 knockdown cells that carry reduced levels of sulfation in their HS and CS chains (Sasaki et al., 2009). Hence, impaired mesoderm differentiation and enhanced neural differentiation phenotypes of several HS mutant ESCs support a role of HS in facilitating BMP4 signaling. Just like HS and its modulation of FGF signaling is critical for initial cell fate commitment, other cell fate decisions during differentiation that are dependent on FGF signaling require HS. For instance, we found that HS, through facilitating FGF signaling, is needed for normal expression levels of the T-box transcription factor Brachyury, which is important for the establishment of mesoderm (Kraushaar et al., 2012). In summary, good evidence was collected showing that HS is a critical component for ESC differentiation into germ-layer type cells through the facilitation of both FGF and BMP signaling to promote mesoderm induction and patterning (Figure 2B).

ESCs abundantly express secreted HSPGs such as perlecan and cell surface syndecans and glypicans. Knockout of perlecan substantially reduces the differentiation potential of ESC-derived mesendoderm progenitors into Pdx1⁺ pancreatic cells (Higuchi et al., 2011). The exact molecular mechanism by which HS modulates pancreatic differentiation remains unknown but may involve interactions between perlecan and pancreas-inducing growth factors such as VEGF and/or FGF (Higuchi et al., 2011) (Figure 2B).

Manipulation of stem cell cultures with HS biosynthesis inhibitors and heparinoids

The emerging understanding of the role of HS in ESC biology has prompted ideas to manipulate ESC fate decisions by means of using HS biosynthesis inhibitors and heparin analogues, also called heparinoids. Inhibition of spontaneous differentiation is advantageous

for the derivation and propagation of pluripotent stem cell populations, whether it is for routine ESC culture or *de novo* derivation of ESCs from blastocysts. As discussed previously, loss of HS induces a more naïve ground state and enhances self-renewal of mESCs. Therefore, inhibition of HS synthesis may improve efforts of ESC derivation. Lanner et al. (2010) tested this potential application with sodium chlorate, an inhibitor of HS sulfation, and found that sodium chlorate treatment of blastocysts substantially improved the efficacy of ESC derivation.

The addition of heparinoids to human ESC (huESC) cultures is another good example of improved ESC maintenance through the manipulation of HS-mediated signaling pathways. huESCs are generally maintained in mouse embryonic fibroblast (MEF)-conditioned medium or unconditioned medium that are supplemented with recombinant FGF2, which is required for self-renewal (Dvorak et al., 2005; Levenstein et al., 2006). Dose-dependent addition of heparin, or MEF-derived HS or HSPGs to unconditioned medium will enhance the mitogenic response of huESCs to FGF2 and lower the optimal dose of FGF2 required for huESC maintenance (Furue et al., 2008; Levenstein et al., 2008). The proliferative boost exerted by heparinoids is due to the enhancement of FGF stability in culture medium and due to facilitating FGF cell surface binding (Furue et al., 2008; Levenstein et al., 2008).

Supplementing cocktails of growth factors that direct stem cell differentiation is a commonly used strategy to obtain desired lineage and cell types. Oftentimes, the efficacy of differentiation and homogeneity of cell populations are suboptimal. Given that physiological HS modulates important cell signaling pathways and cell fate, exogenously supplemented heparinoids may also enable us to manipulate cell signaling and direct cell fate choices. Encouraging results have already been obtained using defined heparin in several differentiation regimens. For example, addition of heparin to ESCs grown in N2B27 culture conditions increases the number of Sox1⁺ cells almost twofold and the effect is both concentration and size dependent (Pickford et al., 2011). Similarly, addition of heparin also enhances hematopoietic specification during EB differentiation of ESCs, and the improved differentiation into erythrocytes is dependent on heparin concentration, size, and N- and 6-O-sulfation (Holley et al., 2011). Furthermore, immobilized heparin and HS significantly promote mesenchymal stem cell growth and differentiation into osteogenic, chondrogenic, and/or adipogenic cell types (Uygun et al., 2009).

In summary, several studies have demonstrated the proof of concept of using inhibitors of HS biosynthesis and heparinoids to enhance the maintenance of ESCs as well as to stimulate their differentiation into therapeutically relevant cell types. HS analogues are particularly attractive candidates for stem cell culture and cell type specification as they are stable and available at relatively low cost. However, additional studies with structurally well-defined heparinoids across various differentiation regimens are necessary to forward the improvement and potential use of heparinoids for directed differentiation.

Conclusions and perspectives

HS is a structurally diverse molecule that interacts with numerous proteins and regulates various developmental processes. Despite various studies that have established a role for HS in ESC differentiation, one question that remains is whether additional fine structure requirements exist for cell signaling pathways and ultimately cell fate. A prerequisite to answering this question would be to establish how HS fine structures become remodeled during differentiation and which structures are prevalent at specific stages of development. The use of phage-based antibodies has proven useful in detecting HS structures expressed in tissue as well as by ESC-derived cells. However, antibody detection is limiting as epitopes are oftentimes insufficiently defined and low abundance HS species may not be detected.

Directed differentiation protocols and the generation of homogenous ESC-derived cell types combined with mass spectrometry or NMR will facilitate the identification of novel stage-specific HS structures. Second, it needs to be established whether structure changes are functionally relevant by affecting distinct signaling pathways that are pertinent to the differentiation process. Genetic studies with disruption of additional HS-modifying enzymes will aid in elucidating the physiological importance of HS fine structures. In light of the importance of HS in promoting lineage commitment of ESCs, conditional cell type-specific knockout of additional HS-modifying enzymes will become essential in deciphering the function of HS during cell specification programs at early and late stages of ESC differentiation. Meanwhile, the function of HS in developmental signaling has become clearer; however, the role of their glycoproteins remains obscure. Which glypicans and syndecans are involved in the modulation of specific signaling pathways and do they bind and contribute as co-factors or are they mere presenters of HS chains? Understanding the structure-function relation of HS with growth factors and their carrier core proteins, and their precise roles in stem and progenitor cell fate will allow us to dissect the molecular mechanisms of HS during embryogenesis and development, and will also enable us to translate these findings into applications that will contribute to the directed manipulation of stem cell fate toward phenotypes beneficial for regenerative medicine.

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Figure 1.

HS biosynthesis.

In mammals, at least 20 enzymes specifically participate in HS biosynthesis. HS formation is initiated and elongated by the co-polymerase complex formed by EXT1 and EXT2. Concomitantly with elongation, the HS chain is modified sequentially by Ndsts, C5 epimerase, Hs2st, Hs6sts, and Hs3sts to generate mature HS.

Figure 2.

Role of HS in mESC self-renewal and differentiation.

(A) HS facilitates FGF signaling to downregulate Nanog and establish a PE-like cell type. HS modulates BMP4 signaling and is required for normal Id1 expression. Upon LIF withdrawal, HS facilitates FGF signaling and downregulation of Nanog to allow for multilineage differentiation. (B) HS facilitates FGF and BMP signaling to promote mesoderm induction and dorso-ventral mesoderm patterning, respectively. HS, through enhancement of BMP4 signaling, has inhibitory effects on neural differentiation. Secreted HS modulates BMP4 signaling by increasing ligand stability. Perlecan might facilitate FGF and/or VEGF signaling to mediate ESC differentiation into Pdx1⁺ pancreatic cells.