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REVIEW ARTICLE

# The role of heparan sulphate in development: the ectodermal story

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## SUMMARY

Heparan sulphate (HS) is ubiquitously expressed and is formed of repeating glucosamine and glucuronic/iduronic acid units which are generally highly sulphated. HS is found in tissues bound to proteins forming HS proteoglycans (HSPGs) which are present on the cell membrane or in the extracellular matrix. HSPGs influence a variety of biological processes by interacting with physiologically important proteins, such as morphogens, creating storage pools, generating morphogen gradients and directly mediating signalling pathways, thereby playing vital roles during development. This review discusses the vital role HS plays in the development of tissues from the ectodermal lineage. The ectodermal layer differentiates to form the nervous system (including the spine, peripheral nerves and brain), eye, epidermis, skin appendages and tooth enamel.

## Introduction

Heparan sulphate (HS), a prototype glycosaminoglycan (GAG), is a linear polysaccharide composed of repeating disaccharide units comprised of an *N*-acetylglucosamine (GlcNAc) and a glucuronic acid (GlcA) (Figure 1). Following polymerization, HS chains undergo secondary modifications, which introduce immense structural diversity as a result of varying substitution with sulphate groups and glucuronic acid epimerization (Figure 2). Similar to other GAGs, HS is found in tissues bound to proteins forming HS proteoglycans (HSPGs). HS is structurally related to heparin, a highly sulphated and epimerized form of HS that is restricted to mast cells and synthesized attached to the small core protein (18 kDa) serglycin (Tantravahi *et al.* 1986). HSPGs are ubiquitously expressed in mammalian cells and are present on cell surfaces, in the extracellular matrix (ECM) and basement membranes.

During evolution, HS emerged in the animal kingdom with the eumetazoa, the first animal clade to display tissues organized into germ layers (Gesteira *et al.* 2011a; Yamada *et al.* 2011). HSPGs are vital in many cellular processes, ranging from development through to adult physiology, and changes in HSPGs have been correlated with a plethora of diseases. HSPGs function primarily through interactions with various protein ligands, and the fine structure of HS chains dictates binding specificity. HSPGs are required for the action of many growth factors and morphogens by localizing them to particular sites (creating a morphogen gradient and forming 'storage pools'), by independently activating signalling pathways and by acting as coreceptors participating in the formation of ternary complexes required for the attachment of specific

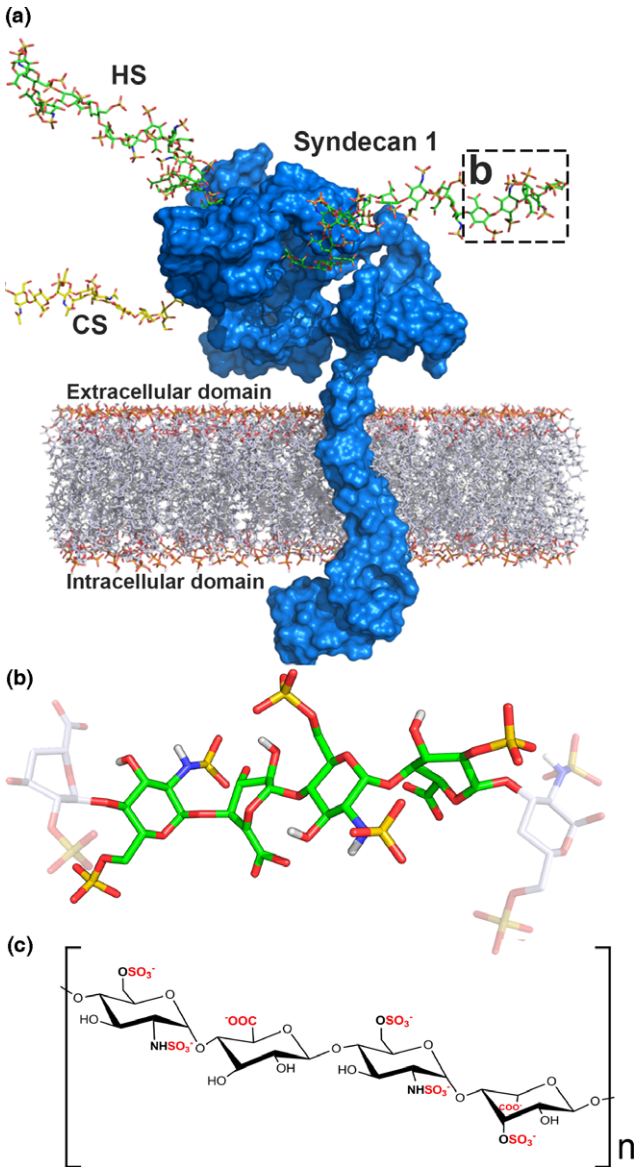


**Figure 1** Proposed model for the HSPG syndecan-1 and HS structure. (a) The core protein of syndecan-1 (blue) is immersed in a lipid DPPC membrane and HS (green carbon atoms) and CS (yellow carbon atoms) chains are attached to the extracellular domain at positions Ser37 (CS), Ser45 (HS) and Ser47 (HS). The structure of syndecan-1 was modelled using MODELLER (Marti-Renom *et al.* 2000) and the DPPC membrane, tetrasaccharide linkage region, and both HS and CS chains were added using charmm version c37b2 (Brooks *et al.* 2009). The highlighted box (b) is represented in more detail in (b). (b) A HS tetrasaccharide is shown in detail with carbon (green sticks), oxygen (red sticks), sulphate (yellow sticks) and nitrogen atoms (blue sticks). (c) Chemical structure of HS, which is comprised of repeating disaccharide units (GlcA or IdoA and GlcN). Putative sulphation and epimerization sites are highlighted in red.

molecules to their receptors. The interaction of HS with growth factors in the ECM also protects these molecules from degradation and regulates their diffusion throughout the tissue (Rosengart *et al.* 1988; Saksela *et al.* 1988).

The ECM is a dynamic and complex environment that plays a critical role during development, and GAGs and PGs are integral components of this matrix. Early in development, three primary germ layers are formed: the ectoderm, mesoderm and endoderm, each with distinct characteristics giving rise to specific tissues or tissue parts. The ectodermal layer differentiates to form the nervous system (including the spine, peripheral nerves and brain), eye, epidermis, skin appendages, tooth enamel and the lining of the mouth, anus and nostrils. As previously mentioned, HSPGs are found both on the cell surface (Figure 1) and in the ECM where they influence a variety of biological processes by interacting with physiologically important proteins, such as morphogens, creating storage pools, generating morphogen gradients and directly mediating signalling pathways, thereby playing vital roles during development (Vlodavsky *et al.* 1991). In this review, we discuss the role of HS in the development of tissues of ectodermal origin.

## *Glycosaminoglycan biosynthesis*

GAG biosynthesis is conserved from nematodes to man and occurs in a non-template-driven manner. GAG biosynthesis is a dynamic enzymatically regulated process which yields chains with a plethora of variable sulphation patterns (Prydz & Dalen 2000). HS/HEP biosynthesis requires a 'primed' core protein upon which the exostosin proteins EXT1/EXT2 polymerize the sugar chains. The core protein is 'primed' by the addition of a tetrasaccharide linkage region. The addition of this tetrasaccharide starts with the addition of a xylose by xylosyltransferase in the late endoplasmic reticulum and/or the cis-Golgi compartment (Bourdon *et al.* 1987; Vertel *et al.* 1993; Gesteira *et al.* 2011b). Thereafter, two galactose monosaccharides (Galb1,4) are sequentially added to the xylose residue by galactosyltransferases I and II (Lohmander *et al.* 1989), and the tetrasaccharide is completed by the addition of a glucuronic acid residue by glucuronosyltransferase I (Vertel *et al.* 1993; Gesteira *et al.* 2011a). The subsequent addition of *N*-acetylglucosamine by *N*-acetylglucosaminyltransferase-I (GlcNAcT-I) determines the fate of the growing GAG to HS/HEP (Prydz and Dalen 2000). Thereafter, biosynthesis continues with the alternating addition of glucuronic acid (GlcA) and *N*-acetylglucosamine (GlcNAc) residues by the HS polymerases exostosin 1 and exostosin 2 (EXT1 and EXT2) (Kitagawa & Sugahara 2000; Senay *et al.* 2000). The structure of this backbone composed of repeating GlcA–GlcNAc disaccharide units is then modified by a group of enzymes, which leads to the great heterogeneity shown by HS/HEP. *N*-deacetylase/*N*-sulfotransferase (NDST) removes the acetyl group from GlcNAc which is subsequently sulphated by the addition of a sulphate group into GlcNS (Aikawa & Esko 1999; Gesteira *et al.* 2011b, 2013). Evidence exists to suggest that the *N*-deacetylation/*N*-sulphation of GlcNAc precedes other modifications, such that NDST1 knockout mice also present a decrease in 2-*O*-sulphation to the same magnitude as *N*-sulphation (Aikawa & Esko 1999; Ledin *et al.* 2004). C5

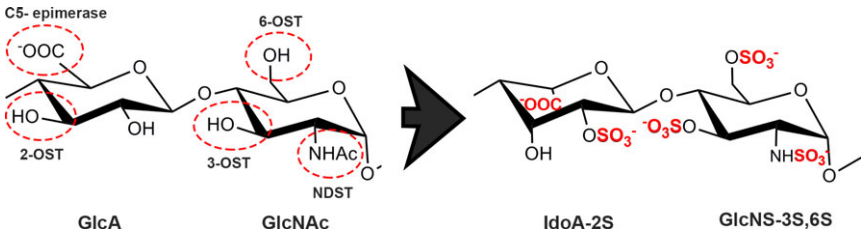


**Figure 2** Schematic of modifications on heparan sulphate chains by biosynthetic enzymes. HS is synthesized by sequential addition of alternating GlcA and GlcNAc by EXT1 and EXT2 respectively. Thereafter, a group of enzymes further modifies the HS chain: C5 epimerase epimerizes GlcA to IdoA, and the sulfotransferases NDST, 2-OST, 3-OST and 6-OST add sulphate groups.

epimerase epimerizes GlcA to α-L-iduronic acid (IdoA), 2-*O*-sulfotransferase sulphates uronic acid, and both 6-*O*-sulfotransferase and 3-*O*-sulfotransferase add a sulphate group to glucosamine residues (Kitagawa *et al.* 1997). A strong interaction has been demonstrated between C5 epimerase and 2-*O*-sulfotransferase, and this association is essential for epimerase stability and translocation to the Golgi apparatus (Pinhal *et al.* 2001). Different isoforms exist for many of the modification enzymes. Four isoforms have been identified for NDST, namely NDST-1–4. The different isoforms present different spatial–temporal distribution patterns. NDST-1 and NDST-2 are more widely distributed with some overlapping tissue distribution, while NDST-3 and NDST-4 have more restricted tissue distribution (Grobe *et al.* 2002). A variety of complete systemic knockout mice and also conditional knockout mice models have been developed for the different biosynthetic enzymes. In essence, HS and HEP originate from the same core structure; however, downstream modifications differentiate the two, resulting in different degrees of epimerization and levels of sulphation. As such, HEP is synthesized upon a serglycin core protein, presents most of GlcA epimerized to IdoA and is heavily sulphated (Kolset & Gallagher 1990; Kolset *et al.* 2004). The downstream modifications are illustrated in Figure 2. The HS chain sulphation pattern is further modified postsynthesis through the action of two extracellular endosulfatases, HS 6-*O*-endosulfatase 1 (SULF1) and 6-*O*-endosulfatase 2 (SULF2) (Lai *et al.* 2003; Kleinschmit *et al.* 2010; Higginson *et al.* 2012). SULFs are present in the extracellular space, and therefore, induction of their expression culminates in further alterations in the HS chain sulphation pattern. The biosynthetic pathway ensures the HS chains present a great variety of sulphation patterns which dictate their binding specificity to different morphogens (Figure 2). Often these specific sulphated motifs are interspersed with unsulphated regions, NS and NA domains respectively (Figure 3) (Rapraeger *et al.* 1994; Davies *et al.* 2003). Moreover, the degree of sulphation also confers different charges (Figure 3B) and levels of rigidity to the HS chains (Figure 3C,D), which also influences protein interactions (Gallagher *et al.* 1992). Torsion angles of the glycosidic linkages dictate chain flexibility, which consequently determines the optimal conformation for binding morphogens (Figure 3C) (Perkins *et al.* 2014). Non-sulphated domains such as GlcA–GlcNAc (NA domains) have been shown to have higher torsion angles than highly sulphated domains such as Ido2S-GlcNS6S (NS domains) due to the fact that the negative charges introduce greater repulsion (Figure 3C) (Perkins *et al.* 2014). Consequently, the NA domains flanking NS domains introduce conformational flexibility to the HS chains (Figure 3D). Thus, the level and pattern of sulphate residues along HS chains dictate their activity (Bishop *et al.* 2007). The binding of growth factors to HS chains present in the ECM creates storage pools, gradients and regulates growth factor diffusion, while when growth factors bind to specific HS chains on cell surface HSPGs, these proteoglycans function as cofactors for triggering signalling pathways, aid in the assembly of signalling complexes, directly trigger signalling pathways and/or may lead to the clearance of growth factors (Bishop *et al.* 2007; Coulson-Thomas *et al.* 2014).

### *Heparan sulphate proteoglycans*

With the exception of hyaluronan (HA), GAG synthesis requires a core protein upon which the biosynthetic process may take place, and thus HS is present in tissues bound to a core protein forming HSPGs. Two main groups of cell surface HSPGs have been described to date: the syndecans and glypicans. Syndecans are comprised of syndecan-1, syndecan-2, syndecan-3 and syndecan-4, which are a family of transmembrane proteins and play an important role in cell–matrix interactions. The syndecan family has also been shown to directly trigger intracellular signalling complexes. The cytoplasmic domain of syndecan-4 binds specifically to phosphatidylinositol 4,5-bisphosphate and phosphatidylinositol 4-phosphate, consistent with findings that protein kinase C colocalizes with focal adhesions (Couchman *et al.* 2002). The phosphorylation of syndecan-4 at Ser183 leads to dramatic conformational changes which serve as a molecular switch regulating the cellular functions of this molecule, such as ablation of the interaction site with syntenin (Koo *et al.* 2006). The other main group of cell surface HSPGs, the glypicans (GPCs), consists of six members: GPC1–6. GPCs are anchored to the membrane of eukaryotic cells by covalent linkage of their C-terminus to glycosylphosphatidylinositol (GPI) (Brown & Waneck 1992). Similar to syndecans, GPCs also act as coreceptors in several signalling pathways and have been shown to play a vital role in biological processes such as cell division, differentiation and

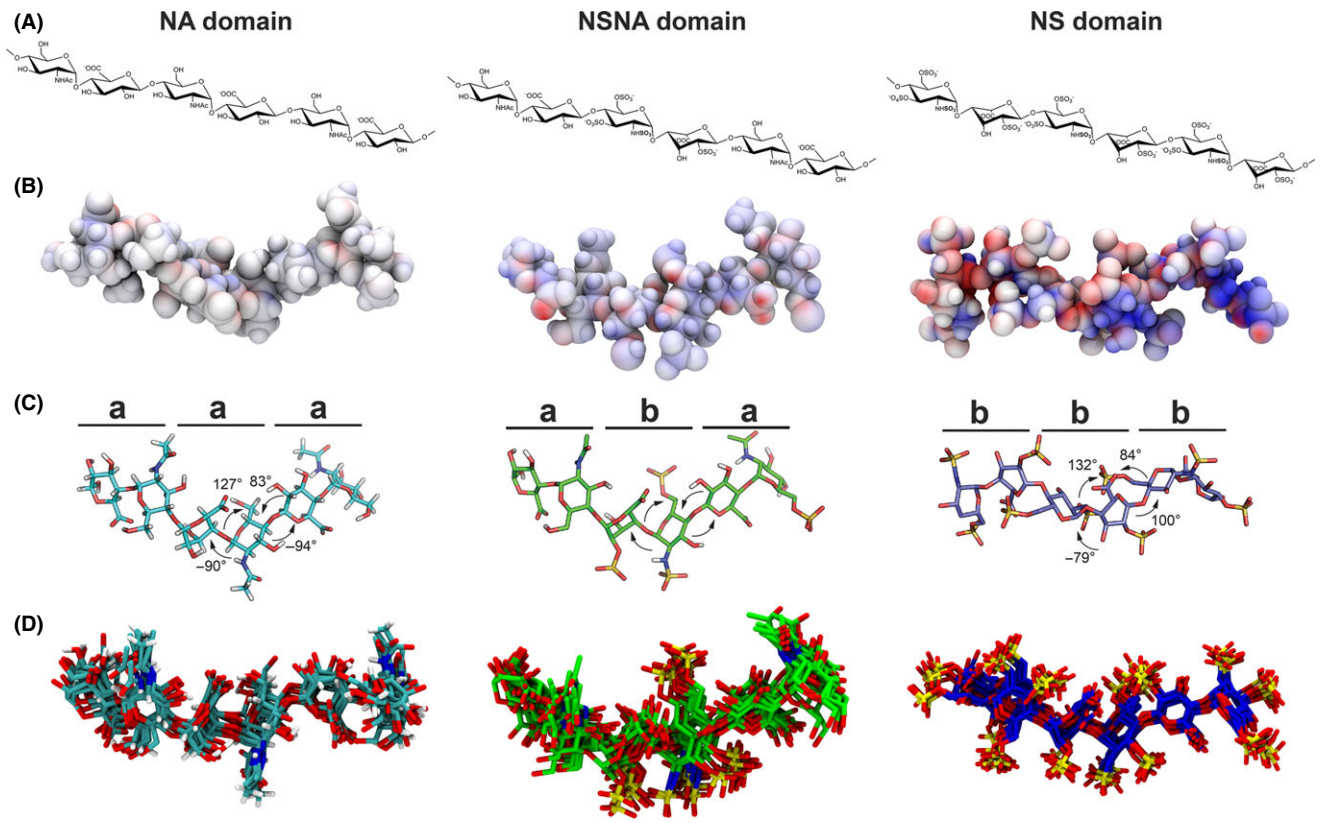


**Figure 3** HS chemical and conformational structures. (A) The chemical structure of HS. HS is comprised of repeating disaccharide units (GlcA or IdoA and GlcN) and may display sulphated regions interspersed with unsulphated regions, creating specific domains, namely NS (N-sulphated), NA (N-acetylated) and NA/NS domains. (B) Electrostatic potential map of the structural domains of HS as calculated by APBS (Baker *et al.* 2001), demonstrating that the more negatively charged NS domain presents high electrostatic potential and the NA domain presents lower electrostatic potential (visualized as normalized volume contour at +12.0 kT/e in blue and −12.0 kT/e in red). Potential isosurfaces were visualized in VMD (Humphrey *et al.* 1996). (C) A representation of the crystallographic torsion angles φ–ψ of the glycosidic linkage of HS, which dictates the flexibility of the differently charged HS domains. The highly electrostatic NS domain (b) presents lower torsion angles in comparison with the less electrostatic NA domain which has higher torsion angles and is consequently more flexible than the NS domain (a) (Perkins *et al.* 2014). (D) Different HS domains after 20 ns molecular dynamics simulations show conformational changes of the HS domains over time revealing the reduced flexibility of NS domains in comparison with NA domains (supporting the unpublished data of Tarsis F. Gesteira). NA domains are represented as cyan sticks, NANS domains are represented as green sticks, and NS domains are represented in purple. The computer simulations and analysis were performed at the Ohio Supercomputer Center (OSC, 1987).

developmental morphogenesis. GPCs have been described as regulators for the Wnt, hedgehog (Hh), fibroblast growth factor (FGF) and bone morphogenic protein (BMP) signalling pathways.

HSPGs are also present in the ECM, such as perlecan, agrin and collagen XVIII, which are all important components of basement membranes. Perlecan is a large multidomain HSPG with a core protein of 470 kDa, comprised of five distinct protein modules. Perlecan has angiogenic bivalency, containing both pro- and anti-angiogenic properties (Willis Chris, *et al.*, 2003; Iozzo *et al.* 2009). Agrin is also a multimodular HSPG that may undergo alternative splicing, which confers additional complexity (Stetefeld *et al.* 2004). Collagen XVIII contains 10 interrupted collagenous domains flanked by non-collagenous regions located at the N- and C-termini (Iozzo *et al.* 2009). Upon cleavage in the ECM, perlecan, agrin and collagen XVIII generate active fragments which modulate cell behaviour (Schulze *et al.* 1996; Marcelo & Bix 2015).

### *Knockout models for heparan sulphate and heparan sulphate proteoglycans*

The first studies to demonstrate the importance of HS during development were done with the *Drosophila melanogaster* (*D. melanogaster*) mutants sugarless (Binari *et al.* 1997; Hacker *et al.* 1997; Haerry *et al.* 1997) and sulphateless (Lin & Perrimon 1999; Lin *et al.* 1999). These pioneer studies demonstrated that HS is required for major signalling events. The sulphateless mutant, which lacks the only known *Ndst1* in *D. melanogaster*, displays disrupted wingless (Wg), Hh and FGF signalling (Lin & Perrimon 1999;

Lin *et al.* 1999; The *et al.* 1999). Currently, genetic knockout mouse models have been generated for most of the enzymes involved in HS biosynthesis and also for the major core proteins, thereby providing invaluable information on the role HS and HSPGs play in mammalian development. Given the importance of HS, many of the animal models are embryonic lethal and therefore many inducible conditional knockout models have also been generated to enable an evaluation of the precise role HS plays in specific cell types and/or tissues.

*Ext1* and *Ext2* complete systemic knockout mouse models are embryonic lethal due to gastrulation defects (Lin & Perrimon 2000; Lin *et al.* 2000), and conditional knockout mice have therefore been generated, $Ext1^{flox/flox}$ (Inatani *et al.* 2003). To date, three complete systemic knockout mice have been generated for *Ndst1* (Fan *et al.* 2000; Ringvall *et al.* 2000; Grobe *et al.* 2005), the latter being a conditional knockout mouse model. Systemic knockout of *Ndst1* in the three *Ndst1* knockout strains leads to perinatal death. The loss of *Ndst1* results in a reduction in *N*-sulphated HS by approximately one-third, which culminates in a 50% decrease in overall HS sulphation (Humphries *et al.* 1999; Ringvall *et al.* 2000; Ledin *et al.* 2004). Thus, these studies provide *in vivo* evidence that the sulphation pattern of HS plays a vital role in the control of cell signalling events. The systemic knockout mouse model has also been developed for Ndst 2, $Ndst2^{-/-}$, and these mice are viable and fertile, however fail to synthesize sulphated heparin by mast cells which express primarily the NDST 2 isoform (Forsberg *et al.* 1999). Knockout models for the sulfotransferases, for example $Hs2st^{-/-}$ (Bullock *et al.* 1998), $Hs2st1^{flox/flox}$ (Stanford *et al.* 2010), $Hs6st1^{flox/flox}$ (Izvolsky *et al.* 2008) and $Hs3st1^{-/-}$ (HajMohammadi *et al.* 2003), and for HS GlcUA C5 epimerase $Hsepi^{-/-}$ (Li *et al.* 2003) have also been generated. Systemic knockout mice for the endosulfatases SULF1 and SULF2, $Sulf1^{-/-}$ and $Sulf2^{-/-}$ mice, have also been generated and the single-knockout mice do not display any obvious abnormalities (Forsberg *et al.* 1999) while the combined knockout mice, $Sulf1^{-/-}$;$Sulf2^{-/-}$, present neonatal lethality due to skeletal abnormalities and kidney hypoplasia (Ai *et al.* 2007; Holst *et al.* 2007; Ratzka *et al.* 2008).

Various knockout mouse models have been generated for the core protein in an attempt to elucidate the role of different PGs in development and pathogenesis. In general, the PG knockout mouse models display mild phenotypes in comparison with those targeting the HS chains, potentially indicating compensatory mechanisms, such as among the syndecan family members. The potential functional overlap or compensatory mechanism among syndecan PGs is currently being investigated in compound knockout studies. Syndecan-1 and syndecan-4 knockout mice, $Syndecan\text{-}1^{-/-}$ and $Syndecan\text{-}4^{-/-}$, are viable and fertile with subtle skin and corneal wound healing defects. $Syndecan\text{-}4^{-/-}$ mice have been shown to display impaired angiogenesis (Echtermeyer *et al.* 2001). $Glypican\text{-}2^{-/-}$ mice are also viable and fertile with no obvious phenotype (Lander & Selleck 2000). On the other hand, $glypican\text{-}3^{-/-}$ mice have severe phenotypes with perinatal death (Cano-Gauci *et al.* 1999). The functions of perlecan and agrin have also been investigated with the use of systemic knockout mouse models. $Agrin^{-/-}$ mice also present a subtle phenotype, and these mice are viable and fertile (Erickson & Couchman 2000). Interestingly, $Perlecan^{-/-}$ mice present severe defects including prenatal death, around embryonic day 10–12, due to rupture of basement membranes in the heart and brain defects, such as exencephaly (Arikawa-Hirasawa *et al.* 1999; Costell *et al.* 1999). Animals that survive embryonic lethality ultimately develop chondrodysplasia, which leads to perinatal death (Arikawa-Hirasawa *et al.* 1999). Therefore, since $perlecan^{-/-}$ mice are embryonic lethal or in some cases die just after birth due to skeletal dysplasia, a mouse model has been developed combining the systemic knockout with conditional site directed overexpression of perlecan, thereby overcoming the severe bone/cartilage defects and enabling the mice to survive into adulthood. Combining collagen 2α1-driven overexpression of perlecan with systemic loss of perlecan is enough to overcome the cartilage abnormalities rescuing perinatal lethality in these compound transgenic mice (Xu *et al.* 2010). Some groups have opted to overexpress perlecan in order to unveil its role in organogenesis to overcome the issues with perinatal death of the $Perlecan^{-/-}$ mice. For example, conditional overexpression of perlecan using the keratin 5 promoter (which drives perlecan expression in epithelial cells) was used to determine the function of epithelial-derived perlecan in odontogenesis (Ida-Yonemochi *et al.* 2011).

## Heparan sulphate regulates signalling pathways and is required for morphogen gradient formation during development

Both CS and HS have previously been shown to modulate the FGF, Wnt/Wg and Sonic hedgehog (SHH) signalling pathways (Ai *et al.* 2003; Nadanaka *et al.* 2008; Dani *et al.* 2012; Coulson-Thomas *et al.* 2014). 6-*O*-sulphated HS is required for FGF receptor dimerization and tyrosine kinase activation (Guimond *et al.* 1993; Pye *et al.* 1998), and as such the selective removal of sulphate groups from the 6-O position by the endosulfatase SULF1 has been shown to inhibit FGF2- and FGF4-induced mesoderm formation (Wang *et al.* 2004). FGF4 and FGF8 ligands are retained in the extraembryonic ectoderm by cell surface-tethered HS chains, and the spatiotemporal expression of cell surface-tethered HS chains has therefore been shown to regulate local FGF signalling activity during mammalian embryogenesis (Shimokawa *et al.* 2011). HSPGs containing a GPC5 core protein and 2-*O*-sulpho-iduronic acid residues at the non-reducing ends of the glycans have been shown to be coreceptors for SHH, and promote SHH binding and signalling in the developing nervous system (Witt *et al.* 2013). Perlecan has also been shown to interact with SHH and is important for SHH function, particularly in the brain (Palma *et al.* 2011). In *D. melanogaster,* the coreceptors for Wg include the GPC division abnormally delayed (Dally)

and Dally-like protein (Dlp) and, interestingly, Dlp is a co-receptor with biphasic activity that can activate or repress Wg signalling, depending on the relative expression levels of Dlp and the Wg receptor, frizzled 2 (Yan *et al.* 2009).

Many developmental regulators bind to HS by means of HS-/HEP-binding domains (HBDs). SHH, for example, interacts with HSPGs via an N-terminal Cardin-Weintraub motif, which is characterized by a cluster of basic amino acids that allows for electrostatic interaction between the positive charges on the protein and the negatively charged sulphates on HS (Rubin *et al.* 2002). The ability of SHH to diffuse through HS-containing ECM is due to N-terminal SHH processing which targets and inactivates this HS-binding motif (Ohlig *et al.* 2012). bFGF has been shown to specifically bind only to the HS chains of syndecan-1 and syndecan-4, and not to the CS chains of these hybrid PGs, although the CS chains are required for the formation of a ternary complex that transfers the growth factor to the corresponding cell surface receptor more efficiently in comparison with HS chains alone (Deepa *et al.* 2004). In the case of Wg, this regulator interacts with the Dlp core protein and the GAG chains enhance this interaction (Yan *et al.* 2009).

HSPGs regulate FGF gradient formation in the developing embryo, which is responsible for the positional identity of tissues, and this role of HSPGs has been reviewed by Balasubramanian and Zhang (Balasubramanian & Zhang 2015). The PGs Dally and Dlp are both essential for Wg gradient formation in *D. melanogaster* wing disc during development (Tsuda *et al.* 1999). However, Dally and Dlp have different roles, and it is the combined actions of these proteoglycans that results in the Wg concentration gradient, by means of a restricted diffusion mechanism (Han *et al.* 2005). Overexpression of the HS 6-*O*-endosulfatase SULF1 has been shown to inhibit the binding of Wg to Dally (Kleinschmit *et al.* 2013). On the other hand, the knockdown of HS 6-*O*-sulfotransferase (Hs6st) and SULF1 in *D. melanogaster*, which have opposite effects on HS sulphation, both increase Wg at the synaptic interface, but have opposite effects on synapse development, that is decreased and increased neurotransmission respectively (Dani *et al.* 2012). SULF1 has been shown to be a positive regulator of Wnt signalling; this cell surface sulfatase which removes 6-*O* sulphates from HS chains decreases the affinity of HS towards the Wnt ligand, thereby promoting the binding of Wnt to the receptor frizzled (Ai *et al.* 2003). In a *Sulf1* knockdown *Xenopus laevis* model, it has been shown that SULF1 is required for normal dorsoventral gradient distribution of SHH in the ventral neural tube (Ramsbottom *et al.* 2014). In *Sulf1* and *Sulf2* knockout mice, cell survival and neurite outgrowth (via FGF2, GDNF and NGF) are impaired in the postnatal cerebellum, demonstrating the importance of HSPG sulphation patterns, while SHH, which determines the laminar organization of the cerebellar cortex, is not affected (Kalus *et al.* 2015). The binding of SHH to PGs in mice has been shown to be required for proliferation of neural stem/precursor cells, but not for tissue patterning (Chan *et al.* 2009).

## The role of heparan sulphate in the development of ectodermal tissues

### *Eye morphogenesis*

The eye originates from both ectodermal and mesodermal tissues (Osipov & Vakhrusheva 1975). Eye development begins with the formation of the optic vesicle, as an outgrowth from the early brain, which eventually makes contact with the surface ectoderm and becomes the optic cup (Pei & Rhodin 1970; Buse & de Groot 1991). The retina, ciliary body, iris and optic nerves originate from the neuroepithelium tissues, while the lens and eyelid originate from the surface ectoderm. Development of the cornea also requires mesenchymal cells and neural crest cells, which invade the corneal stroma after condensation (Pei & Rhodin 1970). Therefore, corneal tissues have multiple origins: surface ectoderm (corneal epithelium), neural crest (corneal endothelium and Descemet's membrane) and mesoderm (corneal stroma and Bowman's membrane). Given that the *Ext1*, *Ext2* and *Ndst1* complete systemic mutants are embryonic lethal, the precise role of HS in eye development requires the use of inducible systems.

The important role that HS plays in eye development has previously been demonstrated using a conditional knockout system with breeding of $Ext1^{flox/flox}$ with *Wnt1-cre*. This system uses site-specific recombinase technology, where Cre recombinase is expressed and removes *Ext1* solely in *Wnt1* expressing cells. The *Wnt1-cre;*$Ext1^{flox/flox}$ mice develop severe corneal defects: corneal endothelium defects, corneal stroma hypoplasia, eyelid closure failure and iridocorneal angle dysgenesis (Iwao *et al.* 2009b). This work demonstrates that HS mediates TGF-β2 signalling required for neural crest cells to form the anterior chamber. Analysis of the embryos from the complete systemic *Ndst1* knockout mice further revealed the importance of HS for eye development. These mice display altered HS sulphation patterns and suffer from coloboma, microphthalmia, anophthalmia, primarily on the C57BL background. Further evidence of the importance of HS, more specifically HSPGs, in eye development came from a gain of function study with agrin which led to developmental eye defects (Fuerst *et al.* 2007). Agrin overexpression leads to anophthalmia, failure of vitreous vessel regression, fusion of anterior chamber structures and optic stalk coloboma, which may lead to the misdifferentiation of the optic stalk as the retina (Fuerst *et al.* 2007). Moreover, loss of sulfotransferase function also leads to defective eye development. For example $Hs2st1^{-/-}$ mice have iris coloboma (Bullock *et al.* 1998); and complete systemic $Ndst1^{-/-}$ mice have severe eye developmental defects, which correlate with impaired SHH and FGF signalling. Interestingly perlecan (HSPG2) exon 3 knockout, which lack the three glycosylation attachment sites for potential HS side chains, have microphthalmia (Rossi *et al.* 2003).

*Cornea.* Limited studies have investigated the role of HS in the development of the anterior chamber of the eye. A number of studies have elucidated the importance of HSPGs, specifically of syndecan-1 and perlecan, in corneal wound healing. However, the precise role HS plays in corneal development is limited by the availability of Cre-driver transgenic mouse lines that would be able to specifically remove HS from the cornea early enough during development. For example, we used the conditional knockout model *Ext1*$^{flox/flox}$*;Kera-cre* in an attempt to elucidate the role HS plays in stromal development, removing *Ext1* from keratocan$^{+}$ cells (keratocytes). However, these mice presented no stromal phenotype (Coulson-Thomas *et al.* 2015). Keratocan expression during mouse corneal development starts around embryonic day 12, and *Ext1* expression would therefore only be ablated from the keratocytes of these mice approximately around embryonic day 13. Potentially slow turnover of the stromal ECM could have hampered the stromal phenotype. However, the important role HS plays in corneal epithelial development was clearly demonstrated using *K14rtTA;TetOcre* removing Ext1 from keratin 14$^{+}$ cells (corneal epithelial cells) (Coulson-Thomas *et al.* 2015). We have recently demonstrated that HS is essential for corneal epithelial stratification (Coulson-Thomas *et al.* 2015). The loss of epithelial HS in the cornea leads to a loss of tight junctions, culminating in corneal dysgenesis (Coulson-Thomas *et al.* 2015).

Previous studies have demonstrated the importance of syndecan-1 and perlecan for corneal wound healing. For these studies, the authors used complete systemic knockout mice for syndecan-1 (*Syndecan-1*$^{-/-}$) and systemic perlecan knockout mice with rescued cartilage expression (*Hspg2*$^{-/-}$*-Tg*), which prevents perinatal death due to premature cartilage development (Arikawa-Hirasawa *et al.* 1999; Costell *et al.* 1999). These complete systemic perlecan knockout mice, which express recombinant perlecan solely in cartilage driven by the cartilage-specific Col2a1 promoter, survive into adulthood. *Syndecan-1*$^{-/-}$ mice undergo normal eye development, but present corneal epithelial defects upon wounding, including delayed re-epithelialization and failed activation of epithelial cell proliferation after wounding (Stepp *et al.* 2002). The altered epithelial cell migration rates may be overcome by seeding *Syndecan-1*$^{-/-}$ cells on fibronectin- or collagen-coated culture dishes, indicating that the epithelial-related defects of *Syndecan-1*$^{-/-}$ mice could be in part due to defects in the basement membrane. Keratinocytes obtained from *Syndecan-1*$^{-/-}$ mice and grown in culture are more proliferative, more adherent and migrate more slowly in comparison with their wild-type counterparts (Stepp *et al.* 2007). Later Stepp and collaborators have suggested a mechanism by which the loss of syndecan-1 affects wound healing. Corneal stromal cells isolated from *Syndecan-1*$^{-/-}$ mice show a decrease in the size of focal adhesion complexes, decreased integrin activation and reduced assembly of fibronectin into fibrils, and consequently migrate faster (Stepp *et al.* 2010). Therefore, the loss of syndecan-1 mediated integrin function leads to changes in matrix assembly, which affect cell adhesion, spread and migration of corneal stromal cells. On the other hand, perlecan knockout mice, Hspg2$^{-/-}$-Tg, do have developmental eye defects, such as microphthalmos. In the cornea, perlecan is primarily present in the basement membrane, and consequently mutant mice lacking this HSPG have a thinner corneal epithelium with a significantly reduced number of cell layers (Inomata *et al.* 2012).

*Sulf1*$^{-/-}$ and *Sulf2*$^{-/-}$ mice have been used to investigate the role of HS in corneal development (Lamanna *et al.* 2007; Rosen & Lemjabbar-Alaoui 2010; Maltseva *et al.* 2013). The roles of SULF1 and 2 were investigated using a corneal wound healing model. This group did not describe any developmental defects that result from ablating SULF1, SULF2 or both SULF1 and SULF2 from the corneal epithelium. However, similarly to *Syndecan-1*$^{-/-}$ mice, *Sulf1*$^{-/-}$ mice present delayed wound healing, while *Sulf2*$^{-/-}$ do not have wound healing defects (Maltseva *et al.* 2013). Interestingly, our studies with *Ndst1*$^{flox/flox}$ mice using the *K14rtTA;TetOcre* driver to remove NDST1 from the corneal epithelium resulted in a more subtle phenotype than *Ext1*$^{flox/flox}$*;K14rtTA;TetOcre* (Coulson-Thomas *et al.* 2015). The corneal phenotype of *Ndst1*$^{flox/flox}$*;K14rtTA;TetOcre* resembled that of *Sulf1*$^{-/-}$ mice (Coulson-Thomas *et al.* 2015). The complete loss of HS in *Ext1*$^{flox/flox}$*;K14rtTA;TetOcre* mice hinders the formation of a stratified epithelium and drastically delays corneal epithelial cell resurfacing after wounding leading to stromal damage (Coulson-Thomas *et al.* 2015).

*Lens.* FGFs are fundamental signalling cues during early stages of lens development and HS is essential for FGF diffusion, gradient formation and morphogen–receptor interaction. Moreover, *N*-sulphation is essential for the binding of FGF2 to HS (Turnbull *et al.* 1992). Complete systemic *Ndst1* knockout mice present disrupted lens determination gene expression and the absence of lens invagination, which leads to severe lens hypoplasia or anophthalmia (Pan *et al.* 2006). A testament to the importance of HS for FGF signalling during lens development is that genetic disruption of either FGFR1, FGFR2 or FGFR3 leads to no developmental lens defects (Huang *et al.* 2003; Garcia *et al.* 2005; Zhao *et al.* 2006). The loss of the HS glycosylation site on perlecan (exon 3) leads to alterations of the lens capsule structure (Rossi *et al.* 2003). These lens defects become more severe when these mice are further bred with *Col18α1*$^{-/-}$ mice, which indicates that HS chains could also have a structural function during lens development (Rossi *et al.* 2003).

*Lacrimal gland.* Extensive studies have demonstrated the important role HS plays in lacrimal gland morphogenesis. More importantly, the HS/FGF signalling axis dictates vital cues for lacrimal gland formation. *Ndst1* expression at the tip of the lacrimal gland bud has been shown to be required for lacrimal gland development, and the loss of NDST1

leads to limited lacrimal gland outgrowth and, in some mice, no lacrimal gland budding. Complete systemic *Ndst1* knockout (*Ndst1*$^{-/-}$) and conditional knockout of *Ndst1*$^{flox/flox}$ with the *LE-Cre* transgenic driver (*Ndst1*$^{flox/flox}$*; LE-Cre*) were used to demonstrate that NDST1 expression is required in lacrimal gland epithelial cells and not in mesenchymal cells. Moreover, NDST1 modifications of HS chains are required for FGF10–FGFR2b complex formation, but not for FGF1–FGFR2b (Pan *et al.* 2008). Another study has demonstrated that both FGF7 and FGF10 are dependent on HS for the gradient formation required for lacrimal and salivary gland morphogenesis. FGF7 has been correlated during gland development with the budding of prebranched epithelial buds, while FGF10 is primarily correlated with epithelial bud elongation, with both events requiring HS. Interestingly, a single amino acid mutation in the FGF10 binding pocket, which reduces its binding affinity to HS, turns FGF10 into a functional mimic of FGF7, and thereby this mutant form of FGF10 leads to lacrimal gland budding rather than elongation (Makarenkova *et al.* 2000). The interaction between FGF10 and FGFR2b and HS was further investigated using knockout mice for HS 2-*O*-sulfotransferase, *Hs2st*$^{flox/flox}$*;LECre*, and 6-*O*-sulfotransferase, *Hs6st1*$^{flox/flox}$*;LECre;Hs6st2*$^{-/-}$, to modify the fine structure of HS (Qu *et al.* 2011). Both *Hs2st*$^{flox/flox}$*;LECre* and *Hs6st1*$^{flox/flox}$*;LECre;Hs6st2*$^{-/-}$ mice presented lacrimal gland hypoplasia; however, mice lacking 6-*O*-sulfotransferases presented a more severe phenotype with strong FGF10 genetic interaction, and finally, the triple knockout mice, *Hs2st*$^{flox/flox}$*;Hs6st1*$^{flox/flox}$*;LECre;Hs6st2*$^{-/-}$, presented the most severe phonotype with abolished lacrimal gland development and disruption of FGF10–FGFR2b–HS complex formation on the cell surface (Qu *et al.* 2011). This study demonstrates that *O*-sulphation of HS is also required for FGF signalling. Lacrimal gland development starts with the formation of the lacrimal gland bud, which invades the FGF10 expressing mesenchyme (Makarenkova *et al.* 2000; Entesarian *et al.* 2005). The lacrimal gland bud invades the mesenchyme until E15.5 when secondary branching initiates to create a complex tubuloalveolar structure. As mentioned above, NDST1 expression by epithelial cells at the tip of the invading bud is required for lacrimal gland morphogenesis (Pan *et al.* 2008). However, a study also elucidated the role of HS, more specifically HS sulphation, in the FGF-producing mesenchyme during lacrimal gland morphogenesis. Mesenchymal ablation of GAGs leads to excessive diffusion of FGF10, which culminates in a loss of FGF signalling response and, consequently, stunts lacrimal gland budding in the presumptive epithelium (Qu *et al.* 2012). This work further demonstrates that lacrimal gland budding requires mesenchymal expression of NDST1 and NDST2, and not Hs2st, Hs6st1 and Hs6st2, to create a FGF10 gradient and limit its diffusion (Qu *et al.* 2012).

*Retina.* HS is also required for retinal development, and early on, HSPGs were found to be expressed in neurites of retinal neuronal cells and are potentially involved in synaptogenesis (Chai & Morris 1994). Changes in the fine structure of HS lead to retinal axon targeting defects in *Xenopus laevis* (Irie *et al.* 2002). HS is expressed throughout retinal development and is required for retinal ganglion cell axon projection towards the optic nerve head (Ogata-Iwao *et al.* 2011). Regional expression of 2- and 6-*O*-sulfotransferase culminates in specific HS species required for axon guidance towards the tectum (Irie *et al.* 2002). Moreover, Netrin-1 and Slit-mediated intraretinal RGC axon guidance require HS (Ogata-Iwao *et al.* 2011). The role of HS, more specifically of HS fine structure, in retinal ganglion cell axon projection to the optic disc was investigated using *Ndst1*$^{flox/flox}$*; Ndst2*$^{-/-}$*;Six3-cre* and *Hs2st*$^{flox/flox}$*;Hs6st*$^{flox/flox}$*;Six3-cre* compound mice. The compound mutants exhibit normal retinal neurogenesis and optic fissure closure; however, these mice present defective optic disc and stalk development leading to optic nerve hypoplasia and aplasia (Cai *et al.* 2014). *Ndst1*$^{flox/flox}$*;Ndst2*$^{-/-}$*;Six3-cre* and *Hs2st*$^{flox/flox}$*; Hs6st*$^{flox/flox}$*;Six3-cre* mice express undersulphated HS chains in Six3-expressing cells, including in the retina, hypothalamus and layer 4 of sensory cortical areas (Furuta *et al.* 2000). These mice present retinal ganglion cell axon projection misrouting in part due to the loss of FGF signalling (Cai *et al.* 2014). *Ext1*$^{flox/flox}$*;Wnt1-cre* mice present disrupted neural crest cell migration due to disturbed TGF-β2 signalling (Iwao *et al.* 2009a). Moreover, *Ext1*$^{flox/flox}$*; Dkk3-cre* mice, which lack HS synthesis in retinal progenitor cells (Dickkopf-3$^{+}$ cells), display severe intraretinal guidance errors, ectopic axon penetration through the neural retina, optic nerve hypoplasia and disturbed centrifugal RGC axon projection towards the optic nerve head (Ogata-Iwao *et al.* 2011).

### *CNS morphogenesis*

The ectodermal tissue differentiates to form the tooth enamel, epidermis and skin appendages and finally the nervous system, including the spinal cord, peripheral nerves and brain. Early studies had already suggested that PGs play an important role in neurite outgrowth, neuronal cell adhesion and differentiation (Oohira *et al.* 1994; Margolis *et al.* 1996). However, the vital role HS plays in the development of the central nervous system (CNS) was first demonstrated with genetic studies using *D. melanogaster*. Wg, the Wnt homologue gene in *D. melanogaster*, is vital for determining segment polarity during embryonic development (Sharma *et al.* 1973; Klaus & Birchmeier 2008). The sugarless and sulphateless *D. melanogaster* mutants, which have impaired HS biosynthesis, have revealed that HS is required for Wg signalling (Binari *et al.* 1997; Hacker *et al.* 1997; Haerry *et al.* 1997; Lin *et al.* 1999). Moreover, syndecan and glypican have been demonstrated to be required for morphogen gradient formation and for mediating signalling cues for CNS histogenesis and axon guidance. Both Wg and Hh distributions require glypican during CNS development (Han *et al.* 2004; Johnson *et al.* 2004; Franch-Marro *et al.* 2005; Hacker *et al.* 2005) and Slit–glypican-1 interactions govern

histogenesis during certain stages of CNS development (Liang *et al.* 1999). The Slit–Robo signalling axis has been shown to require syndecans for midline axon guidance (Hu 2001; Johnson *et al.* 2004; Steigemann *et al.* 2004). FGF-HS signalling pathways are also required for CNS development. *D. melanogaster* mutants for the perlecan homologue present reduced FGF signalling and larval brain neuroblast cell cycle arrest (Voigt *et al.* 2002; Park *et al.* 2003). Thereafter, genetic studies using *Caenorhabditis elegans* (*C. elegans*) HS and HSPG mutants have further demonstrated the important role of HS in CNS development. In *C. elegans*, mutations in the HS biosynthetic machinery and core proteins result in drastic effects on ectoneuronal development and axonal guidance demonstrating that the fine structure of HS has information necessary for CNS development (Kinnunen *et al.* 1998; Bulow & Hobert 2004; Rhiner & Hengartner 2006; Bulow *et al.* 2008; Edwards & Hammarlund 2014).

The role HS plays in the development of the mammalian CNS remains to be fully elucidated. All syndecans-1–4 have been shown to be expressed in the mammalian CNS (Bandtlow & Zimmermann 2000); however, syndecan-3 is the most highly expressed syndecan in nervous tissues during early postnatal development (Carey 1996). HS synthesis and secondary modifications are tightly regulated during mammalian CNS development. Expression of the major HS biosynthetic polymerases, EXT1 and EXT2, has been detected during CNS development in mice from E9.5 and peaks in the cerebral cortex during the early postnatal period (Inatani & Yamaguchi 2003). As with *D. melanogaster* and *C. elegans*, HS is required for FGF, BMP, Wnt and Hh signalling during mammalian CNS development. Embryonic stem cells fail to differentiate into Pax6-positive neural precursor cells in *Ext1*$^{-/-}$ mice due to disrupted FGF and BMP signalling (Kraushaar *et al.* 2012). The 6-O-sulphation pattern, number of sulphates and chain length of HS have been shown to change in the developing neuroepithelium, which functionally correlates with an altered ability to activate FGF2 and FGF8 signalling (Brickman *et al.* 1998). This same group later demonstrated that there is differential expression of HS sulfotransferases in the developing brain, leading to the distribution of variant HS species which orchestrate FGF and FGFR signalling complexes (Ford-Perriss *et al.* 2002). *Ext1*$^{flox/flox}$*;Nestin-Cre* leads to severe nerve patterning defects encompassing those of HS-binding morphogens. HS is required for midline axon guidance and *Ext1*$^{flox/flox}$*;Nestin-Cre* mice present caudal midbrain–cerebellum malformations, small cerebral cortex, loss of major commissural tracts, commissural axon pathfinding defects and lack of the olfactory bulb (Inatani & Yamaguchi 2003; Matsumoto *et al.* 2007). *Ext1*$^{flox/flox}$*;Wnt1-Cre* mice also present commissural axon pathfinding defects (Matsumoto *et al.* 2007). HS, more specifically 2-O-sulphated HS, is required for the formation of signalling complexes between HS, FGF2 and FGFR1, and consequent Erk1/2 activation at the developing telencephalic midline (Chan *et al.* 2015). This same group demonstrated that 2-O- and 6-O-sulfotransferases generate a HS-containing environment which regulates Erk signalling, which is conducive with corpus callosum development (Clegg *et al.* 2014). The important role that GAGs, including HS, play in CNS function and pathogenesis has recently been reviewed (Smith *et al.* 2015).

### *Skin morphogenesis*

The skin serves many important roles, such as serving as the first barrier against environmental insults (requiring survival and regeneration) and also providing the necessary cues for skin appendage formation. The development of skin and skin appendages, including teeth, hair and nails/claws, is regulated by the formation of a placode which requires reciprocal interactions between epidermal and mesenchymal tissues. The skin is composed of the epidermis followed by the basement membrane and the ECM-rich dermis (of mesenchymal origin). The skin basement membrane is composed of a PG-rich specialized ECM. It has been suggested that the skin basement membrane, specifically at the dermal–epidermal junction and hair follicle epithelium, is primarily secreted by epithelial cells (Yamane *et al.* 1996). Skin integrity requires the formation of anchoring complexes by the basement membrane, such as hemidesmosomes, which are required for linking the epidermis to the dermis. HS side chains have been demonstrated as integral players in the assembly of anchoring complexes (Iriyama *et al.* 2011b). Moreover, HSPGs at the epidermal–dermal junction regulate epidermal differentiation and proliferation and are essential for maintaining epidermal homeostasis (Iriyama *et al.* 2011a; Behrens *et al.* 2012).

As mentioned above, *Syndecan-1*$^{-/-}$ mice are healthy and fertile. *Syndecan-1*$^{-/-}$ mice present increased skin epithelial cell proliferation rates, but do not display any morphological changes to their skin or increases in epithelial thickness (Stepp *et al.* 2002). However, upon injury S*yndecan-1*$^{-/-}$ mice present impaired wound healing. *Syndecan-1*$^{-/-}$ mice display proliferation delays after full thickness wounds leading to prolonged hypoplasia, but differently to the corneal epithelium, no migration defects are observed (Stepp *et al.* 2002). *Syndecan-4*$^{-/-}$ mice are also healthy and fertile with normal skin development; however, fibroblasts isolated from *Syndecan-4*$^{-/-}$ mice display reduced wound healing rates *in vitro* and *Syndecan-4*$^{-/-}$ mice present impaired wound healing. In the skin of naïve mice, syndecan-4 is expressed exclusively by epidermal cells; however, upon injury, there is an increase in syndecan-4 expression throughout the dermis. Syndecan-1 and syndecan-4 seem to play a synergistic role in wound healing, where syndecan-1 is primarily involved in keratocyte function, re-epithelialization and stratification, while syndecan-4 is associated with fibroblast migration, wound contraction and angiogenesis. Recently, (Gopal *et al.* 2015) demonstrated that syndecans control TRP channels, thereby controlling fibroblast calcium influx, which in turn impacts the phosphorylation status of focal adhesion kinase. This study indicates that syndecan regulation of TRPC-type channels may control myofibroblast phenotype influencing wound repair and fibrosis. Perlecan

has also been demonstrated to be essential for epidermal formation. Precocious epidermal cell apoptosis causes incomplete stratification in human *in vitro* engineered skin with perlecan-deficient keratinocytes, which leads to a thin and poorly organized epidermis (Sher *et al.* 2006). Further evidence for the role of HSPGs in epidermis morphogenesis has come from studies administering heparanase to skin explants (Vlodavsky *et al.* 1999; Zcharia *et al.* 2005). Moreover, our recent work has demonstrated that knocking out HS in the epidermis leads to epidermal hyperplasia (Coulson-Thomas *et al.* 2014).

*Sweat glands.* Sweat glands, or eccrine sweat glands, are composed of a spiralled intraepidermal duct, a dermal duct comprised of a straight and coiled portion, and a secretory tubule, which is a coiled duct within the dermis. In human embryos, the development of sweat glands begins at ~12–13 weeks primarily on the palms and soles followed by the rest of the body at week 20 (Sato *et al.* 1989), and morphogenesis is only complete after birth. Sweat glands play a vital role in thermoregulation, primarily for cooling and hydrating the skin. Thermoregulation is a vital evolutionary requirement for maintaining homeostasis in endothermal animals, such as horses and camels, where sweating is vital for survival (Schmidt-Nielsen *et al.* 1957; Jenkinson 1973). In contrast to humans that have sweat glands throughout the skin, mice have sweat glands exclusively on the pads of their paws making them more sensitive to extreme temperatures (Lu & Fuchs 2014). In mice, the development of sweat glands commences at embryonic day 17.5–18.5. Initial stages of sweat gland morphogenesis involve epidermal invagination of K14 expressing cells (Sun *et al.* 1979; Moll & Moll 1992), and gradually the inner layer of the developing sweat gland differentiates into luminal cells and expresses K8/K18 in lieu of K5/K14 (Lu *et al.* 2012). Research on the involvement of HSPGs in sweat gland morphogenesis is scarce. However, we have recently shown that *Ext1* knock out in K14 expressing cells, thereby causing invaginating epidermal cells to be void of HS side chains, induces sweat gland morphogenesis in adult mice (Coulson-Thomas *et al.* 2014).

*Hair follicles.* The hair follicle is a composite organ composed of compartments of both epithelial and dermal origin. Similarly to the development of teeth and nails/claws, the development of hair follicles requires intricate epithelial–mesenchymal interactions between the underlying dermis and epidermis culminating in a dermal signal that induces formation of the follicle placode (Biggs & Mikkola 2014). It has been well established that the ECM composition plays a major role in epithelial–mesenchymal interactions (Watt & Huck 2013). Moreover, the presence of a basement membrane physically separating the epidermis and dermis (mesenchyme) is an indicative that basement membrane HSPGs could play an important role in dictating the initial cues for hair follicle morphogenesis. The spatial and temporal distribution of perlecan changes during hair follicle morphogenesis. All human fetal skin basement membranes contain perlecan, but it is concentrated in the dermal papilla during later stages of hair follicle morphogenesis, coinciding with NCAM (calcium-independent neural cell adhesion molecule), which interacts with HSPGs and is involved in cell–cell adhesion (Kaplan & Holbrook 1994). In mice, hair follicle morphogenesis starts around embryonic day 13 and is completed by postnatal day 20 after which morphogenesis of new hair follicles ceases and the existing hair follicles cycle to maintain hair health. Interestingly, we have recently shown that knocking out HS from the epidermis is enough to remove the inhibitory signals that restrict further follicle placode formation in adult mice, and unrestrained hair follicle morphogenesis takes place in mature mice (Coulson-Thomas *et al.* 2014). Studies have also demonstrated the presence of HSPGs other than perlecan in the hair follicle; hair follicles have been shown to express high levels of syndecan-1 (Bayer-Garner *et al.* 2002; Richardson *et al.* 2009; Coulson-Thomas *et al.* 2014) and also, although to a lesser extent, syndecan-3 (Coulson-Thomas *et al.* 2014). Many morphogens that regulate hair follicle morphogenesis are known to require HS for both triggering signalling cascades and correct tissue distribution. An example of this is SHH, which is known to mediate various developmental processes including hair follicle morphogenesis and cycling (Zhang *et al.* 2007; Chang *et al.* 2011; Ohlig *et al.* 2012). Mice lacking epidermal HS present disrupted SHH distribution with drastically increased deposition in the underlying dermis (Figure 4) (Coulson-Thomas *et al.* 2014).

In mature skin, given the impossibility to develop new hair follicles, healthy hair is made possible by the hair follicle undergoing repeated cycles of growth (anagen), regression (catagen) and quiescence (telogen). Both anagen and catagen require significant remodelling of the surrounding

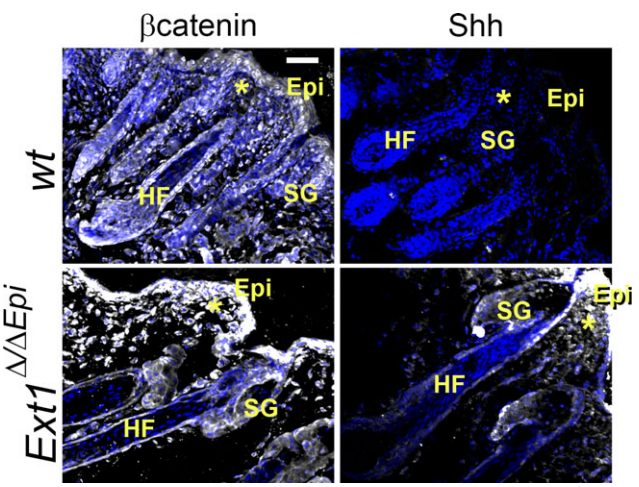


**Figure 4** Loss of epidermal HS disrupts the distribution of morphogens. The knockout of *Ext1* in keratin 14 expressing cells ($Ext1^{\Delta/\Delta Epi}$) leads to a loss of epidermal HS disrupting β-catenin and SHH distribution at the epidermal–mesenchymal junction in comparison with wild-type mice (wt). The epidermis (Epi), a sebaceous gland (SB), a hair follicle (HF) and the underlying dermis (*) are shown in the figure.

ECM to enable growth and regression of the hair follicle. Of significant importance is the matrix remodelling that enables the downward growth of anagen hair follicles, which requires enzymatic degradation of the surrounding ECM. Heparanase expression has been reported during both anagen and catagen, primarily in the inner root sheath (Malgouries *et al.* 2008). Moreover, heparanase inhibition induces cycling into the catagen phase (Malgouries *et al.* 2008). Interestingly, our work knocking out *Ext1* and consequently HS from the epithelial compartment of the hair follicle inhibited cycling into catagen, therefore sequestering all follicles in anagen (Coulson-Thomas *et al.* 2014). Also, heparanase overexpression increased hair growth rate during the anagen phase further supporting the notion that removal of HS chains is required for downward growth during anagen (Vlodavsky *et al.* 1999; Zcharia *et al.* 2005).

*Sebaceous glands.* The sebaceous glands produce sebum, which plays a vital role in hair and skin homeostasis. The lack of sebaceous glands and sweat glands, such as in scar tissue, leaves the skin severely dry, fragile and significantly more prone to infection. Sebaceous glands are attached to hair follicles and are formed as part of the same developmental process. Limited research has investigated the significance of HSPGs in sebaceous gland morphogenesis and cycling. However, we have found that the loss of HS expression in sebaceous glands leads to hyperplasia and increased sebum production (Coulson-Thomas *et al.* 2014).

### *Nail morphogenesis*

Nail development begins with the formation of primordial nails, which appear on the dorsal surface of developing distal digits. Surrounding cells form the nail folds, which are succeeded by the nail matrix containing proliferating keratinocytes. Keratinocytes dorsal to the matrix undergo apoptosis, resulting in keratinization of the proximal nail folds forming the nail plates. Many developmental regulators modulated by HSPG play a role in nail/claw development, including Wnt, Shh and bone morphogenetic protein 4 (BMP4) (Hamrick 2001; Cui *et al.* 2013); however, the direct role of HS remains to be investigated.

### *Tooth morphogenesis*

Tooth morphogenesis involves various stages, namely the bud, cap, bell and late bell stages, which are regulated by sequential and reciprocal interactions between the epithelial and mesenchymal tissues (Thesleff 2003). It is during the bell stage that the hard tissue-forming cells of the tooth (odontoblasts and ameloblasts) differentiate at the interface of the epithelium and mesenchyme and deposit the dentin and enamel matrices respectively (Thesleff 2003). The distribution of basement membrane PGs has been shown to change during cell differentiation and matrix secretion in the developing tooth (Thesleff *et al.* 1981; Bronckers *et al.* 1989; Kogaya *et al.* 1990).

HSPG sulphation and desulphation play a role in dentinogenesis; odontogenic cells are highly sulphated on the cell surface and become desulphated during their differentiation to odontoblasts (Hayano *et al.* 2012). *Sulf1*$^{-/-}$;*Sulf2*$^{-/-}$ double null mutant mice exhibit defective dentin phenotypes, whereas single SULF mutants do not show such defective phenotypes (Hayano *et al.* 2012). This is due to the fact that SULF1 and SULF2 are essential for temporally and spatially regulating the 6-*O*-desulphation of odontogenic cells during their differentiation. Desulphation of HS decreases the binding affinity of Wnt10a towards HSPGs, which facilitates the binding of Wnt10a to its receptor resulting in activation of the Wnt signalling pathway and upregulation of dentin sialophosphoprotein expression, which is a dentin-specific matrix protein that plays a role in the formation of mineralized dentin (Hayano *et al.* 2012). During cementogenesis, the period in which dental follicular cells penetrate the ruptured Hertwig's epithelial root sheath (HERS) and differentiate into cementoblasts, there is an upregulation of the endoglucuronidase heparanase by HERS cells and degradation of the HSPG perlecan in the dental basement membrane, which could result in the release of growth factors such as bFGF bound to perlecan (Hirata & Nakamura 2006). The overexpression of perlecan in epithelial cells in transgenic mice results in an irregular alignment of HERS cells, dull-ended crowns, outward-curved tooth roots and poorly crystallized enamel (Ida-Yonemochi *et al.* 2011). During mouse incisor amelogenesis, syndecans-1–4 are spatially and temporally expressed; syndecan-1 is expressed in undifferentiated epithelial and mesenchymal cells, and syndecan-2, syndecan-3 and syndecan-4 in more differentiated cells (Muto *et al.* 2007). There is a loss of syndecan-4 expression when inner enamel epithelial cells gave rise to ameloblasts (Yan *et al.* 2014). The knockdown of syndecan-4 has been shown to result in reduced cell proliferation and increased expression of amelogenin, ameloblastin, kallikrein 4 and matrix metalloproteinase 20, which are molecules that participate in the formation of enamel (Yan *et al.* 2014).

## Conclusion

A plethora of information has amounted on the vital role HS plays in the development of ectodermal tissues. A vital role has been established for HS in the development of the CNS, retina, cornea and lacrimal gland, which opens new research avenues in regenerative medicine for targeting HS and HSPG core protein expression. However, further research is necessary to unveil additional roles of HS in CNS, retina and cornea pathogenesis. Furthermore, current research has clearly revealed that HS plays a vital role in hair follicle morphogenesis; however, the precise mechanism remains elusive and, exciting future research targeting epidermal HS could provide a cure for alopecia. Plenty of research is still required to unveil the precise role of HS and HSPGs in the formation of skin appendages, such as sebaceous glands and sweat glands, teeth and nails. This

research could have important pharmaceutical implications for targeting HS expression.

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