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Heparan sulfate 2-O-sulfotransferase (*Hs2st*) and mouse development

Valerie A. Wilson,[1] John T. Gallagher[2] and Catherine L.R. Merry[2]

[1] *Institute for Stem Cell Research, University of Edinburgh, Edinburgh EH9 3JQ, UK,* [2] *Cancer Research UK Department of Medical Oncology, Christie Hospital NHS Trust, Manchester M20 4BX, UK*

Heparan sulphate 2-O-sulphotransferase (Hs2st) acts at an intermediate stage in the pathway of biosynthesis of heparan sulphate (HS), catalysing the transfer of sulphate from 3′-phosphoadenosine-5′-phosphosulfate (PAPS) to the C2-position of selected hexuronic acid residues within the maturing HS chain. It is well established that 2-O-sulphation within HS, particularly of iduronate residues, is essential for HS to participate in a variety of high-affinity ligand-binding interactions. HS plays a central role in embryonic development and cellular function, modulating the activities of an extensive range of growth factors. Interestingly, in contrast to the early failure of embryos entirely lacking HS, *Hs2st*$^{-/-}$ mice survive until birth, but die perinatally due to a complete failure of kidney formation. The phenotype of *Hs2st*$^{-/-}$ mutant kidneys suggests that signalling between two tissues, ureteric bud and metanephric mesenchyme, is disrupted. We discuss candidate signalling molecules that may mediate this interaction. The HS generated by these mice lacks 2-O-sulphate groups but is extensively modified above wild type levels by O-sulphation at C-6 of glucosamine-N-sulfate (GlcNS) residues. We will discuss the potentially altered role of this atypical HS in growth factor signalling.
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Introduction

The heparan sulphate proteoglycan (HSPG) family comprises some of the most abundant proteins on the cell surface, and various HSPGs are also common within the extracellular matrix. A growing number of key morphogenic factors are now known to depend on HSPGs for their transport, diffusion, and interaction with signalling receptors [1]. Because of this, HS is likely to be a significant factor in the precise spatio-temporal regulation of differentiation during development [1–3]. The mechanism of this regulation is not yet fully understood. However, the discovery that signalling via members of the Wingless (Wg), hepatocyte growth factor/scatter factor (HGF/SF) and fibroblast growth factor (FGF) families of molecules depends not only upon the presence of HS chains, but on particular structural modifications within them [4–7] suggest that the sugar sequences and sulphation patterns within HS play a central role in the developing embryo.

HS, unlike its structural analogue heparin, exhibits a high degree of structural heterogeneity. This is observed both in the variety of disaccharide units present within the chain, and in the organization of these saccharides into sulphated regions of varying sizes. Two critical characteristics of HS biosynthesis make this possible. One is that no template is used, with the patterns within HS generated by interactions between the biosynthetic enzymes, the core HSPG protein and the nascent chain itself. The other is the tissue-specific and temporally regulated expression pattern of the enzymes that catalyze this process [8,9].

One of these enzymes, 2-O-sulphotransferase (Hs2st), brings about the transfer of a sulphate group to C-2 of hexuronic acids. Iduronic acid (IdoA) residues sulphated at C-2 are a universal component of HS, and have been found in varying amounts in all naturally occurring HS chains characterised to date [10]. Extended sequences of IdoA(2S)-GlcNS form a backbone to define the sulphated (or S-domains) within many of the HS-types studied [11–13]. These repeating units can then be further modified by addition of 6-O-sulphate and, less frequently, by addition of 3-O-sulphate groups to the GlcNS resides. In one study, the HS from 3T3 fibroblasts was found to contain a relatively simple S-domain pattern, of the general structure GlcA-GlcNS-(IdoA(2S)-GlcNS)$_n$-IdoA-GlcNAc in which n ranged from 1–7 [12]. 6-O-Sulphation was present in only a minority of these sequences, being located mainly on centrally positioned

To whom correspondence should be addressed: Catherine L.R. Merry, Cancer Research UK Department of Medical Oncology, Christie Hospital NHS Trust, Manchester M20 4BX, UK. E-mail: cmerry@picr.man.ac.uk

GlcNS residues to form tri-sulphated disaccharides (IdoA(2S)-GlcNS(6S)). Thus, 6-O-sulfotransferases (Hs6st) do not act randomly within S-domains, suggesting that the location of 6-O-sulphates is essential for normal HS function. The almost uniform pattern of IdoA(2S)-GlcNS within HS S-domains has led to the suggestion that N-sulphation and 2-O-sulphation may together form the background on which the chemical imprints of 6-O- and 3-O-sulphation necessary for specific interactions are imposed [14]. The widespread occurrence of 2-O-sulphation has made the specific assessment of the role of this component in HS-ligand interactions very difficult to delineate. For example, HS-derived oligosaccharides can be separated according to their ligand-binding characteristics. Selection for a particular pattern of 6-O-sulphation may also unavoidably select heavily 2-O-sulphated oligosaccharides, merely because the appropriate pattern of 6-O-sulphation only occurs within these regions. This problem is usually overcome by the generation of chemically 2-O-desulphated heparin that will allow the role of 6-O, 3-O and N-sulphation to be investigated in isolation. However, it is not currently possible to remove 2-O-sulphation completely from these preparations [15] resulting in a significant drawback for addressing the function of 2-O-sulphation against the background of a normal HS macrostructure. These problems have been overcome by the generation of a mouse mutant for Hs2st [16], with the additional benefit that the mouse allows the investigation of the role of this specific modification *in vivo*. It is also possible that HS from *Hs2st* mutants may provide an opportunity to investigate unusual cell-specific HS species. *Hs2st* expression levels show extensive variation during development of some tissues, such as the early embryonic heart, showing very low or undetectable levels of *Hs2st* [16]. There may therefore be specific locations within the developing mouse embryo where functional HS is synthesised with little or no 2-O-sulphation. It is obviously of great benefit to study mutations affecting HS biosynthesis and presentation at the cell surface and extracellular matrix. One method of creating mouse mutations, gene trapping, has yielded a number of such mutations.

The secretory gene trap

Gene trapping in embryonic stem cells is a powerful insertional mutagenesis approach that simultaneously allows investigation of novel expression patterns and function of genes in the developing embryo [17]. A commonly used strategy employs a vector containing a splice acceptor 5′ to an in-frame fusion of a *lacZ* reporter gene and selectable marker, (together termed ßgeo), which lacks a translation initiation codon [18,19]. This allows the selection of in-frame vector integrations in the introns of actively transcribed genes, and enables endogenous gene expression to be visualised using 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) staining. A vector-borne polyadenylation sequence prematurely truncates the endogenous transcript. A modified gene trapping approach allows further screening of gene trap integrations for sequences encoding transmembrane or secreted proteins [20]. This uses the empirical finding that a modified ß-galactosidase, containing an in-frame, N-terminal transmembrane domain, is only active if integration occurs downstream of an endogenous N-terminal signal sequence or type II transmembrane domain. This secretory gene trap technique was used to generate a lethal recessive insertional mutation in the gene encoding Hs2st [16] and has subsequently been used in a collaborative venture between U.C. Berkeley and U.C. San Francisco to create an extensive series of mutations (http://ist-socrates.berkeley.edu/∼skarnes/resource.html). Embryonic stem cells and mice found on the list are freely available to academic researchers. Within this list, a surprisingly large number are genes that are of specific interest to researchers investigating the role of proteoglycans in development (see Table 1).

Table 1. Genes mutated using secretory trap strategy

	Gene
Core proteins	
(N-terminal signal sequence)	*Agrin* *Glypican-1* *Glypican-3* *Glypican-4* *Perlecan* *Syndecan-4* *Leprecan-1*
Biosynthetic enzymes	
(N-terminal type II TM domain)	*EXT-1* *Hs6st-1* *Hs2st*

Data on genomic sequence is extracted from mouse genome resource (http://www.ensembl.org/Mus_musculus/_). n/a: not available. *Data only available for human agrin.

Out of 189 known genes trapped using this strategy, some 5% are HSPGs or HS biosynthetic enzymes. This large proportion of genes connected with HS is not associated with very large gene size, low exon: intron ratio, or large number of introns, since trapped genes include rather small genes with few exons (Hs6st-1 has only two exons identified over a total size of 38kb); and small coding sequences (syndecan-4 and leprecan-1 coding sequences span less than 20kb) (source: Mouse EnsEmbl genome database http://www.ensembl.org/Mus_musculus/). It is possible that the conformation or localisation of ß-galactosidase with these N-terminal fusions is particularly favourable, or that they are strongly expressed in embryonic stem cells, or there may be as yet unknown integration site biases in the vector.

The site of insertion of the gene trap vector interrupts the Hs2st coding sequence, leading to the fusion of approximately half of the N-terminal coding sequence of Hs2st with ß-galactosidase [16]. This, together with the apparent absence of wild type transcript, suggests that Hs2st is inactivated by the secretory gene trap mutation. As predicted [21–23], Hs2st

mutants lack 2-O-sulphated residues within their HS, reinforcing the idea that Hs2st is a single gene with a unique enzymatic activity. Interestingly HS isolated from *Hs2st*$^{-/-}$ embryonic fibroblasts had a novel composition and structure, quite unlike any HS previously investigated (see below), and its overall design suggested regulative interactions between the HS biosynthetic enzymes. The phenotype of the *Hs2st*$^{-/-}$ mice clearly demonstrates that, for a variety of key developmental events, HS containing 2-O-sulphation is essential. However, we were surprised to observe that although the abnormal *Hs2st*$^{-/-}$-derived HS was compromised in its binding to some growth factors, we could demonstrate that the "mutant" polysaccharide retained the co-receptor function of wild-type HS for FGF-1, -2 and HGF, at least as measured in mitogenesis and migration assays using primary fibroblasts from *Hs2st*$^{-/-}$ embryos.

Patterns of sulphation in mutant HS

While the N-deacetylase: N-sulfotransferases (NDST), 6-O- and 3-O-sulphotransferases all comprise multigene families [24–26], *Hs2st* is likely to encode a unique enzymatic activity, as described above. It was anticipated therefore that the *Hs2st*$^{-/-}$ mouse, unlike the *EXT-1*, *NDST-1*, *NDST-2* and *3-O-sulfotransferase* (*Hs3st*) mutants [27–30] would completely lack one 'step' in the HS biosynthetic pathway. As discussed earlier, this pathway generates a mature HS chain in which sulphated domains occur containing relatively high levels of both N- and O-sulphation. This close relationship between N- and O-sulphation is maintained in the abnormal HS synthesized by *Hs2st*$^{-/-}$ embryos. In HS purified from *Hs2st*$^{-/-}$ embryonic fibroblasts, 2-O-sulphation is completely absent [31], confirming that, at least in these cells, there is only one functional Hs2st. However, this loss of 2-O-sulphation is compensated for, at least in terms of charge, by an increase in both N- and 6-O-sulphation. This increase takes the form of extended S-domains heavily substituted with 6-O-sulphates. It was noticeable that 6-O-sulphates were increased only in the S-domains and not in the flanking "mixed sequences" of alternating GlcNS- and GlcNAc-containing disaccharides. Thus, in the HS of the *Hs2st*$^{-/-}$ mouse, Hs6sts target sulfated domains for extensive modification of the regions where 2-O-sulphate groups are normally found (Figure 1). This finding is in agreement with a previously published study of the alteration of HS structure in a CHO cell line, isolated from a chemical mutagenesis screen that lacks Hs2st function [32]. As the discrete S-domain structure is one of the most conserved and characteristic features of HS [11,33], it is interesting that it is maintained even after removing 2-O-sulphation, one of its major components.

Sulphation patterns govern HS-ligand interactions

As discussed earlier, one of the key characteristics of HS is its domain structure of spatially-discrete sulphated sections (the S-domains) interspersed with flexible regions of low sulphation. This domain organisation has a functional role in generating patterns, both in terms of the sequence of sugars within the sulphated domains, and the spacing between these domains, that determine the capacity of HS to bind and regulate the action of growth factors and morphogens. For example, the optimal binding requirements of HGF/SF and FGF-2 differ, particularly regarding the relative importance of 2-O and 6-O-sulphation

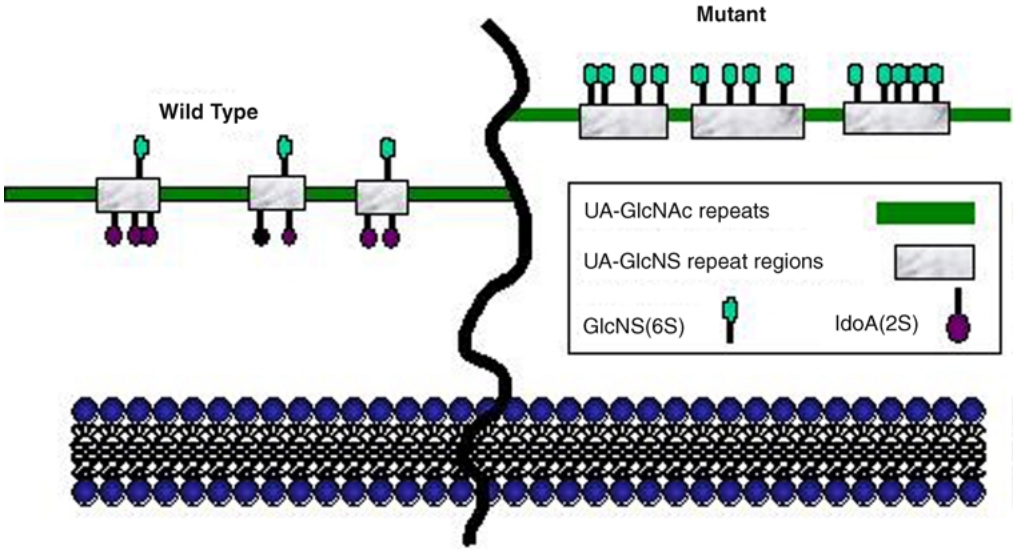

Figure 1. A model demonstrating the differences in content and similarities in organisation in HS from wild type and *Hs2st*$^{-/-}$ fibroblasts. Within the wild type HS, ~16 disaccharides/100 occur within GlcNS-repeat regions. These S-domains contain ~7 2-O-suphated disaccharides and ~3 will be 6-O-sulphated. By contrast, the HS from the *Hs2st*$^{-/-}$ fibroblasts contains ~28 disaccharides/100 within GlcNS-repeat regions and ~16 of these will be 6-O-sulphated with no 2-O-sulphation.

[7,34–36], and these interactions appear to be essential for their biological function.

HS from *Hs2st*$^{-/-}$ mice has a reduced affinity for selected ligands

2-O-Sulphation has been a particular focus of the investigation of the HS-binding of the FGF family in particular. For many of these growth factors 2-O-sulphation is an essential component of the high-affinity binding region within HS [15,35,37–39]. This conclusion was based on studies with chemically modified heparin, and analyses of S-domains of HS selected for high affinity FGF binding. The importance of 2-O-sulphation was re-affirmed by crystallographic studies that identified key interactions with between FGF and the 2-O-sulphate groups [40]. It was therefore expected that HS from *Hs2st*$^{-/-}$ mice would have a significantly lower affinity for FGF-1 and -2 than that from wild type mice, and this was observed experimentally [31]. This could be contrasted with the unaltered binding profiles seen for other HS ligands such as HGF and fibronectin where 2-O-sulphation was not an essential component of the high affinity-binding motif [7,41].

Hs2st$^{-/-}$ fibroblasts can mount a signalling response to FGF-1 and FGF-2

To assess the role of cell surface HS lacking 2-O-sulphation in promoting the formation of a signalling complex with these growth factors, primary fibroblasts isolated from *Hs2st* mutant embryos were used. FGF signalling, assayed by mitogen-activated protein kinase phosphorylation (MAPK), was HS-dependent in these cells. Surprisingly, given the difference in affinity described above, the signalling response of the mutant cells for FGF-1 and FGF-2 was as strong as the wild type [31]. These findings lead to the surprising conclusion that although high affinity binding of these two growth factors to HS does require 2-O-sulphation, this is not essential for potentiation of a signalling response, at least in fibroblast cell cultures. The signalling response to HGF/SF in the *Hs2st*$^{-/-}$ cells was also normal, but this was less surprising because HGF/SF bound with normal apparent affinity to the mutant HS chains.

Although the results from the affinity and signalling studies appear at first to be contradictory, they can be resolved in a number of ways. Firstly, recent evidence indicates that certain sulphation patterns within HS may be able to selectively potentiate the action of discrete FGF/FGF-receptor (FGFR) combinations [42]. For example, 2-O-desulphated heparin can potentiate FGF-1 signalling through FGFR-2(IIIb), but not FGFR-1. Secondly, the gross differences in affinity between various HS types and ligands observed *in vitro* may have far more subtle roles to play *in vivo*, where the binding properties of HS at the cell surface may bring about an increase in local growth factor concentrations in the immediate environment of the signalling receptors. Moreover, a difference in the kinetics of interaction between HS and a particular growth factor may dramatically alter its presentation to receptors on target cells [43]. It is possible that strong binding of HS to a ligand may actually be inhibitory, holding the protein in a position or conformation that prevents it from forming a signalling complex with its receptor. This has been shown for mammary cell HS, which binds FGF-2 with high affinity but fails to activate signalling [44]. Heavily 2-O-sulphated regions of HS deficient in 6-O-sulphation are negative regulators of FGF-1 and FGF-2 [6,35].

The activity seen in the embryonic fibroblasts from the *Hs2st*$^{-/-}$ mice is however unlikely to represent an animal-wide response. The restricted expression patterns of the other modifying enzymes *in vivo* (particularly 6-O-sulphotransferases [25]) suggests that the elevated 6-O-sulphation found in mutant embryonic fibroblasts may not be present in all organs and tissues of the mutant embryos. It is therefore likely that in other tissues the loss of 2-sulphation may not be associated with such a dramatic increase in 6-O-sulphation and in some tissues this may lead to the disruption of particular HS-ligand interactions.

For some HS-dependent factors, the maintenance of an approximately normal domain structure and charge distribution may be more important than the patterns of sulphation within the S-domains themselves. This is likely to be the case for some multimeric cytokines in particular (e.g. platelet factor-4 [45] and interferon-γ [46]) as these have been shown to depend heavily on the correct spacing of S-domains within HS chains, with some additional requirements for specific sulphation patterns for optimal interaction.

This combination of potential responses to the aberrant HS therefore makes the phenotype very difficult to interpret. There will be an altered binding specificity to some growth factors (such as FGF-1 and FGF-2) and not to others (e.g. HGF/SF). In some tissues this may have little effect on the signalling response (for example FGF-2 in fibroblasts) but this will probably not be true of all tissues. A careful examination of the phenotype may therefore help us to better understand the role of correct patterning of HS in development.

Kidney development in *Hs2st*$^{-/-}$ mice

The most dramatic difference between wild type and *Hs2st*$^{-/-}$ mice is the complete lack of kidneys in over 95% of the mutants [16]. As shown in Figure 2, kidney development is the result of reciprocal interactions between the ureteric bud and the metanephric mesenchyme. In *Hs2st* mutants, a normal ureteric bud develops and the initial events of mesenchymal condensation also occur. Mesenchyme specified for nephrogenesis can be observed by the expression of Pax2, which is induced correctly on contact with the ureteric bud [47]. Genes that are expressed in the entire wild type ureteric bud are expressed in *Hs2st*$^{-/-}$ mutants [16]. However, genes that are normally restricted in expression to wild type ureteric bud tips (e.g. the glial cell line-derived neurotrophic factor [GDNF] receptor c-ret) are quickly downregulated after bud outgrowth in mutants, and no evidence of bifurcation is seen [16]. Thus, the Hs2st phenotype appears

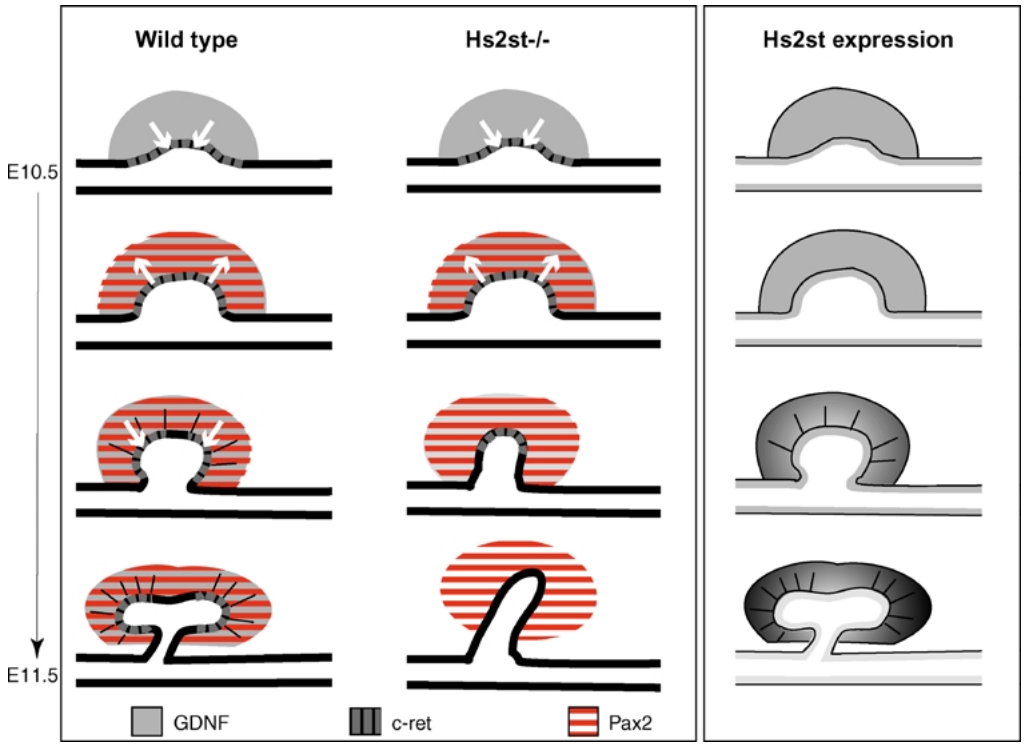

Figure 2. Diagrammatic representation of the *Hs2st*$^{-/-}$ phenotype in early kidney development, together with the expression of Hs2st at these stages. Expression of GDNF, c-ret and Pax2 is shown in wild type and mutant kidneys in the left hand box. GDNF and c-ret are initially expressed in mutants, but thereafter are strongly downregulated. Pax2 is induced, and expression remains in the mesenchyme. Note that Pax2 expression is present throughout the ureter during development in both wild type and *Hs2st*$^{-/-}$ mutant mice (not indicated). However, no further mesenchyme differentiation, evidenced by morphological condensation (radial lines), is apparent. Expression of Hs2st is present in both ureteric bud and metanephric mesenchyme when the Hs2st defect becomes manifest, but it is downregulated in ureteric bud later, while expression increases in the mesenchyme. Arrows: known induction events.

at the time when wild type metanephric mesenchyme first induces ureteric bud branch formation, indicated by the upregulated expression of *ret*. A requirement for sulphated proteoglycan in branching morphogenesis had previously been shown by chlorate treatment of wild type embryonic kidney rudiments, disrupting sulphation of glycosaminoglycans [48]. Chlorate-treated tissues apparently undergo normal nephrogenesis, but ureteric buds fail to branch or grow. This also points to a primary role of sulphated HS (perhaps specifically 2-O-sulphated HS) in ureteric bud branching, rather than metanephric mesenchyme differentiation.

Does this abrupt cessation of kidney morphogenesis suggest the location where wild type HS must be in order to elicit normal kidney development? Hs2st itself is expressed in both ureteric bud and metanephric mesenchyme, when mutants first require it, and thus we cannot exclude one or other tissue as the required source of wild type HS. HS is found throughout the wild type kidney, with particularly high concentrations in the basement membrane between the ureteric bud and metanephric mesenchyme [48]. Ureteric bud cells additionally extend short laminin-rich processes into contact with the metanephric mesenchyme [49]. Thus growth factors passing between ureteric bud and metanephric mesenchyme (and vice versa) cross an HSPG-rich matrix. However, this is not necessarily an obligate route for growth factors in the kidney, which may pass directly from cell to cell.

The implication of HS as necessary for optimal growth factor signalling in other systems has led us to examine the phenotype of mutants that disrupt kidney development where growth factor or receptor signalling is perturbed. While a host of mutants affect kidney development, most act later or earlier than the formation of the first ureteric bud branch. Embryos lacking Emx2, a homeobox-containing transcription factor made by the ureteric bud, show kidney defects that are highly similar to those in *Hs2st*$^{-/-}$ mutants [50]. However there are no known targets of this gene in the kidney, so this mutant is uninformative with respect to a possible requirement for HS in a specific growth factor/receptor interaction in ureteric bud branching.

Two classes of growth factor merit further investigation as possible candidates for this interaction: GDNF, (see Figure 2), and members of the FGF family. Mice lacking functional GDNF, or its receptor, c-ret [51], show identical kidney phenotypes. Most fail to grow a ureteric bud, but a small proportion have an unbranched ureteric bud. Thus, GDNF is required for

initial ureteric bud outgrowth, but in the small number of kidneys that bypass this requirement, a further requirement for branch initiation (i.e. the stage at which Hs2st mutants fail) is revealed. Clearly then, 2-O-sulphated HS is not necessary for GDNF signalling per se, since Hs2st mutants show normal ureteric bud outgrowth. However, we cannot rule out more complex interactions between these molecules. Optimal levels of GDNF signalling appear to be critical: GDNF heterozygotes show a very high incidence of smaller kidneys compared to wild type mice (presumably due to premature cessation of ureteric bud branching) or complete failure of one or both kidneys to form [52–54]. At least in part, this may be due to positive autoregulatory loops involving GDNF and *c-ret* expression, since inhibition of GDNF signalling leads to *c-ret* downregulation. In *Hs2st*$^{-/-}$ kidneys (as in chlorate treated kidneys) [55], *c-ret* expression is extinguished from the ureteric bud, as is GDNF expression from the mesenchyme. Therefore, it is possible that in *Hs2st*$^{-/-}$ mutants, branching fails because a peak of GDNF/c-ret signalling after ureteric bud outgrowth is not achieved. However, in this case one might expect a more variable *Hs2st*$^{-/-}$ phenotype if critical levels of GDNF signalling were reached in some kidneys but not others, as in *GDNF* heterozygotes.

Little information exists concerning the role of FGFs and their receptors in early kidney development. However, widespread expression of a transgene encoding the extracellular domain of FGFR-2(IIIb), thought to act as a dominant negative mutation, leads to very early kidney defects reminiscent of those in *Hs2st*$^{-/-}$ embryos [56]. It should be noted that while this is thought to act by homodimerising with wild type FGFR-2(IIIb), and to a large extent copies the phenotype seen in FGFR mutations, kidney defects are not described in embryos homozygous for targeted mutations in FGFR-2(IIIb) [57]. Therefore, dimerisation with other partners of this soluble receptor fragment may be responsible for the phenotype. Of the other FGFRs that have been documented, *fgfr1(IIIc)* is expressed in the ureteric bud, and its favoured ligands, FGF-1 and FGF-2, are capable of supporting ureteric bud branching and growth in an isolated ureteric bud culture system *in vitro* in the presence of GDNF [58]. In this system, GDNF itself is not capable of inducing branching on its own. Therefore, either GDNF, or a member of the FGF family would seem to be candidates for growth factors whose activity requires 2-O-sulphated HS.

In summary, the role of HS in the localisation of growth factors and modulation of their activity is central for normal embryonic development. Mice that carry mutations in *Hs2st* and in other HS biosynthetic enzymes offer a useful tool for elucidating the mode of action of HS in embryogenesis, and provide tractable models for biochemical and cellular studies.

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