Influencing Hematopoietic Differentiation of Mouse Embryonic Stem Cells using Soluble Heparin and Heparan Sulfate Saccharides

Rebecca J. Holley, Claire E. Pickford, Graham Rushton, Georges Lacaud, John T. Gallagher, Valerie Kouskoff, and Catherine L. R. Merry

From the School of Materials, Materials Science Centre, The University of Manchester, Manchester M13 9PL, United Kingdom and the Cancer Research UK, Paterson Institute for Cancer Research, The University of Manchester, Wilmslow Road, Manchester M20 4BX, United Kingdom

Heparan sulfate proteoglycans (HSPG) encompass some of the most abundant macromolecules on the surface of almost every cell type. Heparan sulfate (HS) chains provide a key interaction surface for the binding of numerous growth factors and morphogens, helping to define the ability of a cell to respond selectively to environmental cues. The specificity of HS-protein interactions are governed predominantly by the order and position of sulfate groups, with distinct cell types expressing unique sets of HS epitopes. Embryos deficient in HS-synthesis (Ext1−/−) exhibit pre-gastrulation lethality and lack recognizable organized mesoderm and extraembryonic tissues. Here we demonstrate that embryonic stem cells (ESCs) derived from Ext1−/− embryos are unable to differentiate into hematopoietic lineages, instead retaining ESC marker expression throughout embryoid body (EB) culture. However hematopoietic differentiation can be restored by the addition of soluble heparin. Consistent with specific size and composition requirements for HS-growth factor signaling, chains measuring at least 12 saccharides were required for partial rescue of hematopoiesis with longer chains (18 saccharides or more) required for complete rescue. Critically N- and 6-O-sulfate groups were essential for rescue. Heparin addition restored the activity of multiple signaling pathways including bone morphogen protein (BMP) with activation of phospho-SMADs re-established by the addition of heparin. Heparin addition of soluble heparin. Consistent with the related GAG heparin is virtually fully sulfated along its length.

Targeted disruption of Ext1 or Ext2 demonstrates the essential requirement for HS during early development, with lethality in HS-deficient embryos prior to E8.5 because of defective gastrulation (1, 2). Although primitive streak formation is initiated by a small percentage of embryos, they fail to form recognizable mesoderm and extraembryonic tissues. Ext1−/− and Ext1+/− ESCs have been obtained from E3.5 blastocysts (1). Following induction of spontaneous differentiation, Ext1−/− ESCs showed a delay or absence of both endoderm and mesoderm markers (1). Furthermore, differentiation of Ext1−/− ESCs into neuroectodermal precursors is defective (3). Recently, ESCs have been derived from Ext1−/−/Ext2−/− embryos and following LIF withdrawal these ESCs showed a complete absence of markers of differentiation (4).

During development all three germ layers are formed from the primitive ectoderm in a process known as gastrulation: epiblast cells are recruited to the primitive streak where they undergo an epithelial-to-mesenchymal transition generating mesoderm and definitive endoderm (5). Newly formed mesoderm subsequently migrates away from the streak and is patterned response throughout embryonic development and adulthood. A key component of this process is the sugar polymer heparan sulfate (HS), which coats the outer surface of almost every cell within the body enabling the selective detection of environmental signals. HS polymerization is catalyzed by a heterodimer consisting of EXT1 and EXT2 enzymes resulting in the addition of alternating glucuronic acid (GlcA) and N-acetylgalactosamine (GlcNAc) residues. This backbone is then enzymatically modified by de-N-acetylation and N-sulfation of selected GlcNAc residues to form N-sulfoglucosamine (GlcNS), epimerization of a subset of GlcA residues to iduronic acid (IdoUA) and the addition of sulfate groups at the O-position of GlcA/IdoUA and 6-O- and rarely 3-O-position of GlcNS/GlcNAc. Modification is highly regulated and not complete, forming a cell-type specific pattern of highly sulfated domains (S-domains) interspersed by unmodified regions with the length, position and patterning of sulfate groups ultimately determining HS:ligand binding. The related GAG heparin is virtually fully sulfated along its length.

Cells have adopted elaborate systems to identify growth factor and morphogen cues in order to elicit an appropriate response throughout embryonic development and adulthood. HS polymerization is catalyzed by a heterodimer consisting of EXT1 and EXT2 enzymes resulting in the addition of alternating glucuronic acid (GlcA) and N-acetylgalactosamine (GlcNAc) residues. This backbone is then enzymatically modified by de-N-acetylation and N-sulfation of selected GlcNAc residues to form N-sulfoglucosamine (GlcNS), epimerization of a subset of GlcA residues to iduronic acid (IdoUA) and the addition of sulfate groups at the O-position of GlcA/IdoUA and 6-O- and rarely 3-O-position of GlcNS/GlcNAc. Modification is highly regulated and not complete, forming a cell-type specific pattern of highly sulfated domains (S-domains) interspersed by unmodified regions with the length, position and patterning of sulfate groups ultimately determining HS:ligand binding. The related GAG heparin is virtually fully sulfated along its length.

The abbreviations used are: HS, heparan sulfate; HSPG, heparan sulfate proteoglycans; ESC, embryonic stem cells; GlcA, glucuronic acid; IdoA, iduronic acid; EB, embryoid body.

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†To whom correspondence should be addressed: Stem Cell Glycobiology Group, School of Materials, Materials Science Centre, The University of Manchester, Manchester M13 9PL, UK. Tel.: 44-0-161-306-8871; Fax: 0161-306-3586; E-mail: Catherine.merry@manchester.ac.uk.
Evidence to suggest that the differentiation defect in Ext1<sup>−/−</sup> cells is, at least in part, due to an impaired response to BMP4, highlighting a critical role for HS as a co-receptor in the BMP4 signaling pathway. Addition of heparin to wild-type cells also altered the outcome of differentiation, suggesting that the application of exogenous GAG offers a novel mechanism for influencing ESC fate.

**EXPERIMENTAL PROCEDURES**

ESC Maintenance and EB Differentiation—Ext1<sup>−/−</sup> and Ext1<sup>+/−</sup> ESCs were provided by Jeff Esko/Dan Wells (University of California, San Diego) (1). Matched 129sv wild-type ESCs were generated in house. ESCs were maintained on irradiated feeder cells, and transferred to gelatin for two passages prior to EB generation as described previously (19). Recombinant mouse Noggin/Fc chimera (0.2 µg/ml, R&D systems, Abingdon, UK) and SB431542 (10 µM, Tocris, Bristol, UK) were dissolved in DMSO and added at day 0 alongside a DMSO alone control. Heparin, HS (pig mucosa, containing both low- and high-sulfated species), CS, DS, selectively desulfated heparin and sized heparin fragments (all from Iduron, Manchester, UK) or organon HS (gift from Organon Co., Oss, The Netherlands) were added at day 0 at 1 µg/ml unless stated. Heparinases I/II/III (Iduron) were used at 5 mIU each.

Gene Expression Analysis—Total RNA was extracted using an RNeasy mini kit including a DNase step (Qiagen, Crawley, UK). 2 µg of total RNA was reverse-transcribed into cDNA using oligo-dT and AMV reverse transcriptase (Promega, Southampton, UK). PCR was performed as described previously (19). For primer sequences see supplemental Table S1.

Immunocytochemistry—Cells were fixed with 4% paraformaldehyde and prepared for staining as described previously (3). Antibodies used are detailed in supplemental Table S2.

Flow Cytometry—Cells were harvested using cell dissociation buffer (Invitrogen, Paisley, UK), and processed for flow cytometry as detailed previously (19). Antibodies used are detailed in supplemental Table S2.

Secondary Methylcellulose Colony-forming Assays—Cells were mixed into methylcellulose-containing medium with specific growth factors and cytokines to promote the growth of either hemangioblast or hematopoietic cell as described previously (19). Blast colonies were scored at day 5 and hematopoietic colonies were counted at day 6 (primitive erythrocytes) and day 9 (definitive precursors containing one or more myeloid lineages with or without erythrocytes). Results are representative of at least three independent experiments.

BMP4 Response Assay—ESCs were plated for 24 h in serum-free KO-DMEM (Invitrogen), before supplementation with 0–20 ng/ml BMP4 (R&D Systems) for 2 h at 37 °C. Cells were washed with PBS and resuspended in lysis buffer (50 mM Tris-HCl, 1 mM EGTA, 1% (w/v) Triton X-100, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.27 M sucrose, 0.1% (w/v) 2-mercaptoethanol, complete protease inhibitor mixture (Roche, Burgess Hill, UK), pH7.5) determining protein concentration using BCA Protein Assay Reagent (Pierce).

Western Blotting—Cells were harvested and protein extracted as above. 20 µg of total protein was separated using
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The flow cytometry profile of Ext1<sup>+/−</sup> ESCs stained with 10E4 was intermediate between that of wild-type and Ext1<sup>−/−</sup>, suggesting a decrease in the number of 10E4-reactive epitopes (Fig. 1C). 3G10 binding following heparinase digest however showed that a similar number of chains to wild-type are present, as only one 3G10-reactive epitope is produced by heparinase activity per chain (Fig. 1D). This difference was explained following GAG purification, whereby Ext1<sup>−/−</sup> HS chains measured only half the length of wild-type, supporting the view that the Ext1 locus is haploinsufficient (1).

HS Is Required for Hematopoietic Marker Expression—The formation of recognizable mesodermal structures fails in Ext1<sup>−/−</sup> embryos and preliminary studies have suggested that Ext1<sup>−/−</sup> ESCs have defects in both mesodermal and endodermal differentiation (1). Therefore using an EB method optimized for the formation of mesodermal/hematopoietic lineages we investigated the ability of Ext1<sup>+/−</sup> and Ext1<sup>−/−</sup> cells to differentiate into hemangioblast and blood progenitors.

Ext1<sup>−/−</sup> cells readily formed EBs in culture (Fig. 2A), although EB size and proliferation rate was decreased compared with wild-type (Fig. 2, A and B). Prior to EB initiation, all three ESC lines displayed high levels of cell surface ESC markers SSEA1, CD31, and cKit (22, 23) (Fig. 2, C and D). In wild-type EBs expression of cKit and CD31 was rapidly downregulated during the first few days of EB differentiation, as ESC identity was lost and differentiation initiated (Fig. 2D). By days 3 to 4, cKit expression was significantly reduced in ~60% of cells, concurrent with the onset of Flk1 expression within the cKit<sup>low</sup> population, marking the hemangioblast (Fig. 2F). CD31 expression was resumed by a proportion of cells by day 6, marking the appearance of hematopoietic and endothelial populations. CD41<sup>+</sup> populations emerge in wild-type cells from EB day 4 to 5 defining the onset of primitive and definitive hematopoiesis (24–26), reaching a maximal at day 6 (Fig. 2, D and E and data not shown). At this stage of differentiation a dual CD34<sup>+</sup>/CD41<sup>+</sup> cell population was apparent, highlighting cells of the definitive hematopoietic progenitor lineage (27). A small percentage of CD41−/CD34<sup>+</sup> cells were also present in EB cultures at day 6, marking cells restricted to the endothelial lineage. Ext1<sup>+/−</sup> cells appear to successfully differentiate in EBs, expressing the same differentiation markers as wild-type, albeit at slightly decreased levels (Fig. 2).

In contrast to wild-type, expression of ESC markers (cKit/CD31/SSEA-1) was retained in Ext1<sup>−/−</sup> cells throughout EB differentiation (Fig. 2, C and D). A slight reduction in CD31/cKit expression was apparent in a small proportion of the EB population at day 4 (Fig. 2D). Nevertheless these proteins were not completely lost from the cell surface and indeed high expression of CD31/cKit was resumed by virtually all EB cells by day 6, suggesting that either ESC marker expression was restored, or that CD31<sup>low</sup>/cKit<sup>low</sup> cells were lost from the culture at later day stages. Cell surface expression of hemoangioblast radiolabeling and purification of GAGs confirming the absence of HS chains.

The results of complementation experiments in Ext1<sup>−/−</sup> cells indicated that the absence of HS chains measured only half the length of wild-type, supporting the view that the Ext1 locus is haploinsufficient (1).

RESULTS

Ext1<sup>−/−</sup> ESCs Lack Cell Surface HS Chains—Ext1<sup>−/−</sup> ESCs were readily maintained in culture forming optically bright colonies with typical ESC morphology, although colonies phenotypically appeared more compact than wild-type, with little visible differentiation (Fig. 1A). Similar to wild-type, Ext1<sup>−/−</sup> ESCs stain positive for ESC marker Oct4 and present a high nucleus to cytoplasm ratio with minimal actin cytoskeleton visible (Fig. 1B).

To confirm the absence of HS chains at the cell surface of Ext1<sup>−/−</sup> ESCs, flow cytometry was performed using HS-specific antibodies. Wild-type cells showed positive staining with antibody 10E4, which recognizes a mixed N-sulfated/N-acetylated epitope (21) and with 3G10, which recognizes unsaturated uronic acid stubs remaining after heparinase cleavage of HS chains (Fig. 1, C and D). Ext1<sup>−/−</sup> cells were negative for both these antibodies with meta-

3 C. L. R. Merry, unpublished data.
blast marker Flk1 and hematopoietic-specific marker CD41 were absent, indicating Ext1⁻/⁻ cells fail to commit to hematopoietic differentiation (Fig. 2, E and F).

**HS Is Required for Hemangioblast-derived and Hematopoietic Colony Formation**—To confirm both the absence of differentiation in Ext1⁻/⁻ cells and the potential of Ext1⁺/⁺ cells to form hematopoietic colonies, EBs were dissociated into single cells and assayed in semi-solid clonogenic replating assays for their ability to form hemangioblast-derived blast colonies at EB day 3.5 or primitive and definitive hematopoietic colonies at EB day 6 (Fig. 2, G and H). Hemangioblast colonies were produced by both wild-type and Ext1⁺/⁺ cells, albeit reduced by ~50% in Ext1⁺/⁺ cultures. No blast colonies were produced by Ext1⁻/⁻ cells, consistent with the absence of Flk1 expression implying a HS-dependent step during the specification of the hemangioblast. This result was mirrored following hematopoietic replating with reduced numbers of colonies in Ext1⁺/⁺ and an absence of recognizable hematopoietic colonies in Ext1⁻/⁻ cultures (Fig. 2H).

Interestingly Ext1⁻/⁻ EBs failed to form large numbers of secondary EBs, which are characteristically generated by undifferentiated cells in these clonogenic replating assays (10). On closer inspection, EB-like structures were apparent during the first few days of secondary culture, yet proliferation was not maintained. Instead at day 5, when colonies were scored, only non-viable indistinct colonies were visible (supplemental Fig. S1).

**ESCs Are Recoverable from Ext1⁻/⁻ EB Cultures**—To further confirm the retention of an ESC-like fate by most Ext1⁻/⁻ cells upon EB formation, Ext1⁻/⁻ and wild-type EBs were also replated at day 6 under ESC growth conditions (+LIF) (Fig. 2, I and J). The majority of recovered wild-type cells (97%) displayed a large flattened differentiated appearance, devoid of Oct4 expression, with an extensive actin cytoskeleton and limited proliferative capacity (Fig. 2, I and J). In contrast, two distinct cell types were visible in Ext1⁻/⁻ cultures; the majority of cells appeared morphologically similar to ESCs with a large nucleus to cytoplasm ratio and were
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Oct4+ (~93%); the remainder were flattened and Oct4−, similar to those seen in wild-type cultures (Fig. 2, I and J). Upon passaging, Oct4− cells were rapidly lost from Ext1−/− cultures, with pure populations of Oct4+ ESC-like colonies maintainable for greater than 10 passages (data not shown).

Heparin Addition to Ext1−/− EBs Restores Hematopoietic Development—As HS appears critical for mesodermal and hematopoietic differentiation, we hypothesized that the addition of soluble heparin may compensate for the lack of cell surface HS on Ext1−/− cells. Thus increasing concentrations of heparin were added to Ext1−/− cells from day 0, and EB cells stained with differentiation markers or replated under hematopoietic-colony forming conditions (with heparin addition) at day 6 (Fig. 3, A and B). With the addition of 10 ng/ml heparin, no rescue of differentiation was observed. However as the concentration of heparin was increased to 100 ng/ml and above, ESC-marker SSEA-1 was down-regulated and expression of hematopoietic marker CD41 became apparent together with an emerging CD34+/CD41+ population (Fig. 3A). Consistent with marker expression hematopoietic colonies were produced in secondary replating assays (Fig. 3B). This effect was dose-dependent, with optimal hematopoietic differentiation seen with the addition of 1 μg/ml heparin (Fig. 3B). Significantly, the number of hematopoietic colonies produced from heparin-treated Ext1−/− cultures was almost identical to those seen with wild-type cells, illustrating the very efficient rescue of hematopoiesis by exogenous heparin. Interestingly, increasing the heparin concentration further in Ext1−/− cells, up to 10 μg/ml, resulted in a decrease in the numbers of hematopoietic progenitors, suggesting that increasing the concentration above a threshold limit becomes inhibitory (Fig. 3B).

Minimal differences were observed in the absolute number of colonies formed by Ext1−/− cells whether heparin was included during secondary replating or not (supplemental Fig. S2), implying that the obligatory HS-dependent step is already fulfilled during formation of CD34+/CD41+ precursors. However heparin addition during replating did enable robust secondary EB formation from control Ext1−/− cultures, although when added at this stage, hematopoiesis was not promoted (Fig. 3B and supplemental Fig. S2).

To confirm rescue of EB differentiation by soluble heparin, further analyses of Ext1−/− EBs treated with 1 μg/ml heparin were performed. Heparin addition restored EB size and proliferative capacity, suggesting heparin addition allows an efficient response to both differentiation and proliferative factors (compare Fig. 3, C and D with Fig. 2, A and B). Down-regulation of c-Kit and expression of Flk1 was also apparent at day 4, marking hemangioblast commitment (Fig. 3E).

RT-PCR was used to track marker expression during differentiation (Fig. 3F). Wild-type cells progressively down-regulate ESC-marker Oct4, show transient expression of Brachyury and Flk1 from days 2 to 3 and express hematopoietic transcription factors SCL, GATA1, and β-Major from days 3 to 4 (Fig. 3F), as seen previously (10). Similar expression patterns were also present in Ext1+/− cultures. Control Ext1−/− EBs without heparin addition failed to down-regulate ESC marker Oct4 and although expression of epiblast marker FGF5 was initiated, its expression was atypical. No significant differences in phosphorylated ERK were detected during the time period of this assay (data not shown) suggesting that other signaling pathways play a more significant role. Similar atypical expression of Brachyury was also seen in Ext1−/− cells, with message apparent from day 2. However unlike in wild-type cultures where expression is transient, becoming down-regulated at later EB stages as later mesodermal markers are induced, Brachyury instead persisted in Ext1−/− cells with an absence of later markers (Fig. 3F). This highlights the failure of Ext1−/− cells to progress beyond the epiblast/early mesodermal stage, instead reverting back to an ESC-like state, albeit with some expression of Brachyury. A small number of Flk1 transcripts were seen at day 6, producing a faint band following RT-PCR (Fig. 3F), however cell surface Flk1 protein was not present at day 6 nor at later day stages by flow cytometry (Fig. 2F and data not shown). Following the addition of heparin to Ext1−/− cultures, wild-type-like expression of FGF5, Brachyury and Flk1, together with hematopoietic markers SCL, GATA1, and β-Major were apparent, albeit slightly delayed, suggesting the restoration of differentiation (Fig. 3F).

To confirm the loss of ESC identity by Ext1−/− EBs grown in the presence of heparin, day 6 EB cells were replated under ESC growth conditions. The resulting cells had a large flattened differentiated appearance (Fig. 3G), failed to express Oct4 (Fig. 3H) and had limited proliferative capacity as seen previously in wild-type cells (Fig. 2J).

Addition of Heparin to HS-Competent Cells Inhibits Differentiation—We also investigated the effect of soluble heparin addition to EB differentiation in a HS-competent environment (Fig. 4). Addition of very low doses of heparin (10 ng/ml) significantly enhanced hematopoietic differentiation of wild-type cells, leading to an increase in the number of CD41+ and dual CD34+/CD41+ definitive hematopoietic cells and subsequently a significant increase in hematopoietic colony formation (Fig. 4, A and B). However as the concentration of heparin was further increased, differentiation was instead inhibited in a dose-dependent fashion, leading to a decrease in the percentage of CD41+ precursors and a reduction in hematopoietic colony development (Fig. 4, A and B). Addition of 1 μg/ml heparin, the optimum concentration for promoting hematopoiesis in Ext1−/− cells, resulted in a ~36% reduction in primitive/definitive hematopoiesis, with a ~70% reduction at 10 μg/ml. Therefore the effect of exogenous heparin addition varies depending on the provision of endogenous HS in the system.

Chain Length and Sulfation Are Critical in Influencing Differentiation—The observation that heparin can rescue hematopoiesis effectively in Ext1−/− cells, provides an attractive system to determine the critical requirement for specific HS epitopes in mesodermal differentiation. Chain length greatly affects the ability of different growth factors to bind to HS with growth factors such as FGF requiring relatively short chains whereas VEGF requires a longer sequence (28, 29). Therefore we selected a panel of heparin oligosaccharide chains of between 6 and 30 saccharides in length and assessed their ability to rescue differentiation at 1 μg/ml.

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FIGURE 3. Heparin addition restores differentiation of HS-deficient Ext1<sup>−/−</sup> ESCs. A, flow cytometry for SSEA-1 and CD34/CD41 at EB day 6 following addition of increasing concentrations of heparin from 0 to 10 µg/ml from day 0. B, hematopoietic replating at EB day 6 following growth with increasing concentrations of heparin. Bars represent S.D. of the mean, and significant values relative to control (no heparin) are indicated *, p < 0.05. C, phase images at EB day 6 following growth with 1 µg/ml heparin. D, final cell number per ml at EB day 6. Starting cell concentration was 5000 cells per ml. * indicates a significance of p < 0.05. E, flow cytometry for cKit/Flk1 at EB day 4 with and without heparin addition at 1 µg/ml. F, RT-PCR during EB differentiation for stem cell marker Oct4, mesodermal transcription factor Brachyury, hemangioblast marker Flk1, hematopoietic transcription factor SCL, and GATA1, globin gene β-Major, and epiblast marker FGF5. β-Actin is the loading control. G and H, phase images (G) and actin/Oct4 staining (H) of day 6 Ext1<sup>−/−</sup> EBs grown with 1 µg/ml heparin, following replated under ESC growth conditions. Results are representative of at least three independent experiments.
A heparin hexasaccharide (dp6, where dp is degree of polymerization of the heparin chain) failed to promote differentiation (Fig. 5A). However a size-dependent increase in the appearance of CD41+/H11001+ hematopoietic precursors was apparent as the size of the chain was increased further. Dodecasaccharide sequences promoted few CD41+/H11001+ cells (7% of total), although addition of dp14 oligosaccharides resulted in a significant 4-fold increase in CD41 expression within the EB resulting in a third of cells expressing CD41. The percentage of CD41+ cells increased further with increasing chain length with maximal CD41 expression apparent when chains measured greater than 18 saccharides in length (Fig. 5A), with addition of dp20 and dp24 sequences resulting in the production of hematopoietic colonies to unfrac- tionated heparin (dp30) (Fig. 5, A and B). Consistently, complete digestion of heparin with heparinases prior to addition abolished rescue (Fig. 5, A and B). Thus, relatively long chains are needed for efficient differentiation, distinct from the relatively short chains shown to be required for FGF signaling (29).

Previously, we have demonstrated that in wild-type cells specific and essential changes in HS-sulfation patterning occur during differentiation (19). Therefore, the number and position of sulfated residues are likely to significantly alter HS:growth factor binding and the efficiency with which it can rescue differentiation. Heparin is sulfated along its entire length, unlike the native cell surface GAG HS which is highly patterned with alternating regions of high/low sulfation. Addition of HS (from pig mucosa, containing both high and low sulfated HS species) restored expression of CD41/CD34 (Fig. 6A), dramatically increased cell proliferation (Fig. 6C), and promoted primitive/definitive hematopoietic precursor formation (Fig. 6D), similar to heparin. This was also true for an overall less-sulfated HS type, although the extent of rescue with this species of HS was reduced (supplemental Fig. S3 and data not shown).

To assess the importance of different sulfation groups along the chain, a range of heparins chemically modified to deplete specific sulfation motifs were tested for their ability to promote hematopoiesis in Ext1−/− cells. No rescue was obtained with selectively N-desulfated, 6-O-desulfated, or fully-desulfated heparin chains (Fig. 6, A and D). However 2-O-desulfated heparin was able to partially rescue hematopoiesis (Fig. 6D).

Related GAGs CS [GlcA (±2S)-GalNAc (±4S±6S)n] and DS [GlcA/IdoUA (±2S)-GalNAc (±4S±6S)n] have both been shown to bind and subsequently promote growth factor signal transduction (30). Thus we asked whether any sulfated GAG, regardless of composition, could rescue differentiation. Interestingly DS was able to partially rescue hematopoiesis in Ext1−/− cells at 1 μg/ml (Fig. 6, A and D). However this could
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not be improved by decreasing/increasing the concentration of DS used (data not shown), suggesting that its effect is limited. CS showed no ability to influence differentiation (Fig. 6, A and D). CS and DS are synthesized by Ext1/H11002/H11002 cells.3 Thus in the absence of HS, both within the Ext1/H11002 embryo and in vitro, native CS and DS are unable to compensate for the deficiency in HS.

Finally these saccharides were assayed for their ability to inhibit wild-type EB differentiation at 1 μg/ml. Only heparin, HS and 2-O-desulfated heparin successfully inhibited differentiation, causing a decrease in hematopoietic colonies by 34, 51, and 40% respectively (Fig. 6E). Fully desulfated heparin caused no inhibition of hematopoiesis; surprisingly instead its addition appeared supportive of EB growth and differentiation, with a 21% increase in CD41 expression and a significant increase in the formation of primitive erythrocyte colonies in secondary replating assays (Fig. 6, B and E). Therefore the type (and likely pattern) of sulfate residues within the heparin/HS chain is critical for the regulation of differentiation.

Heparin Addition Rescues the Expression and Activity of Multiple Growth Factors—Ext1/H11002 cells fail to differentiate to Flk1+/hemangioblasts, suggesting that they lack the competence to adequately initiate and respond to the developmental
signals required for mesodermal and hemangioblast commitment, pathways restored by heparin addition. Multiple signaling factors are implicated, with prominent roles for BMP4, Wnt3/8, and Nodal. Consistent with this, addition of BMP antagonist Noggin and SB431542 (TGFβ-receptor inhibitor) inhibited hemangioblast development of wild-type cells (Fig. 7A).

By RT-PCR transcripts for Nodal, Wnt3a, and Wnt8 are visible in wild-type cultures from day 2 persisting until day 3 or 4 (Fig. 7B). However Wnt3a and Wnt8 were absent in Ext1−/− cells at early day stages, instead being aberrantly induced from day 4/5. Although Nodal message was visible in Ext1−/− cells at day 2, it was later expressed again from day 5 onwards (Fig. 7B).

Wild-type ESCs express BMP4 in agreement with its role in ESC maintenance (31) (data not shown). However BMP4 message is rapidly down-regulated upon differentiation and is low/undetectable in wild-type EBs at day 2 before expression is re-instigated from day 3 onwards at the time of Brachyury/Flik1 induction (Fig. 7B). In Ext1−/− cells no repression of BMP4 is seen upon EB formation with BMP4 expression instead maintained throughout EB differentiation.

Upon addition of heparin to Ext1−/− ESCs, expression of Wnt3a and Wnt8 were restored at earlier day stages, albeit later than in wild-type cells (Fig. 7B). Furthermore, suppression of BMP4 expression at day 2 was initiated suggesting Ext1−/− cells were committing to differentiation, with BMP4 re-expressed from day 4, concurrent with the onset of hemangioblast marker expression.

Considering the prolonged expression of BMP4 message in Ext1−/− cells and the restoration of transient expression upon heparin addition we looked at the downstream targets of BMP signaling to study the activity of BMP ligands expressed during differentiation. In wild-type cells expression of BMP4 is

**FIGURE 7.** Heparin addition to Ext1−/− ESCs restores multiple signaling pathways. A, flow cytometry for Flik1 at day 3.5 in wild-type EB cultures after the indicated treatment. DMSO control at the equivalent concentration as used to dissolve inhibitors is shown. B, RT-PCR analysis for the expression of growth factors BMP5, Nodal, Wnt3a, and Wnt8 during EB differentiation. C, Western blot analysis during EB differentiation for pSMAD1/5/8, Brachyury and Flik1. α-Tubulin is used as a loading control. D, Western blot for pSMAD1/5/8 following addition of increasing concentrations of BMP4 to ESCs. E, Western blot in wild-type EB cultures following addition of 1 µg/ml heparin.
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seen at day 3 and consequently simultaneous phosphorylation of SMAD1/5/8 (downstream from BMP4) was visible, peaking at day 3 to 5 (Fig. 7C). pSMAD1/5/8 activation correlates with up-regulation of Brachyury and Flk1 protein, suggestive of BMP4s significant role in hemangioblast commitment. Although BMP4 message is expressed in Ext1−/− cells throughout EB culture, pSMAD1/5/8 remained low (Fig. 7C). Consistent with RT-PCR results, Brachyury protein was present but delayed, and no Flk1 protein was produced. Therefore although BMP4 message is expressed, no downstream activation of the BMP4 pathway occurs and consequently cells fail to express hemangioblast markers. Following addition of heparin to Ext1−/− cultures, BMP4 expression was stimulated and clear activation of pSMAD1/5/8 at the time of Brachyury/Flk1 induction was apparent (Fig. 7C).

To confirm this decrease in response to BMP4 in the absence of HS, we challenged wild-type and Ext1−/− ESCs with increasing concentrations of exogenous BMP4 and studied pSMAD1/5/8 response (Fig. 7D). A dose-dependent increase in pSMAD1/5/8 was seen in wild-type cultures, although this was strongly decreased in Ext1−/− cells. Response to BMP4 however was recovered in Ext1−/− cells following heparin addition, supporting the need for HS for successful BMP4 signaling.

Addition of heparin to wild-type EB cultures decreased hemangioblast commitment, therefore if HS:BMP4 signaling is important we might expect altered signaling via SMAD1/5/8. In accord with this, pSMAD1/5/8 activation was decreased in heparin-treated wild-type cultures, concomitant with a decrease in Brachyury and Flk1 induction (Fig. 7E).

DISCUSSION

Ext1−/− and Ext2−/− embryos arrest in development prior to gastrulation highlighting the essential requirement for HS during early embryogenesis (1, 2). Ext1−/− ESCs can be maintained under normal ESC growth conditions without significant differentiation. Thus HS is dispensable for the maintenance of ESC self-renewal. Work by Sasaki et al. (32) contradict this finding, stating that RNAi knock-down of Ext1 leads to spontaneous differentiation of ESCs into extraembryonic endoderm. This is in contrast to what we, and others, have observed when HS is completely absent in Ext1−/− cells (3, 4) or when, in our laboratory, Ext1 is down-regulated using RNAi.3 Similarly, no effect on pluri-potency was apparent in NDST1/2−/− ESCs where N-sulfation is absent (33, 34). Indeed we (and others) have previously demonstrated that ESCs express a very low sulfated form of HS at their cell surface and have proposed that this may act to suppress spontaneous differentiation by damping the response to exogenous pro-differentiation signaling cues, many of which are likely to require sulfated motifs within HS (3, 34). Therefore we consider it unsurprising that HS-deficient ESCs remain pluripotent. As soon as differentiation is initiated, rapid up-regulation of HS-sulfation occurs with different sulfation patterns generated depending on the resultant cell lineage (3, 19). We hypothesize that these variations enable each cell type to respond selectively to the unique factors required for the formation of that specific lineage.

Mesodermal and hematopoietic commitment via the formation of EBs is a multi-step process, requiring multiple HS-dependent growth factors, with events within the EB mimicking gastrulation and yolk sac hematopoiesis. Ext1−/− ESCs were able to form EB-like structures under the conditions used, and to subsequently progress to the early mesodermal stage (Brachyury+). However Ext1−/− EBs failed to induce further differentiation markers which would indicate progression to the hemangioblast, or to down-regulate Brachyury transcript/protein at later day stages. This suggests that these cells are able to initiate exit from self-renewal and mesodermal commitment yet the retention of Brachyury expression alongside ESC markers without further hematopoietic progression signifies a block in differentiation and full restriction to mesoderm. ES-like cells (Oct4+ with tight cell-cell adhesions) could also be recovered from late stage Ext1−/− EB cultures, whereas this ability is lost rapidly by wild-type cells suggesting HS-deficient cells are not terminally differentiated. This result mimics the situation in HS-deficient embryos, where Brachyury expression is apparent in some embryos, yet the extent of expression and its overlap with subsequent markers is defective resulting in embryonic lethality (1).

The addition of soluble heparin during EB differentiation effectively rescued the block in hemangioblast commitment observed in Ext1−/− cultures in a dose-dependent manner. Surprisingly, addition of 1 μg/ml, a relatively low concentration of heparin, resulted in the formation of equivalent numbers of blood colonies to wild-type. This suggests that soluble heparin can largely compensate for the lack of correctly positioned cell surface HS in this system, allowing an appropriate response to the HS-dependent growth factors involved, however the timing of differentiation and the extent of rescue varied depending on the type and sulfation status of the heparin/HS chain used. It is also noted that although the hemangioblast/hematopoietic markers Flk1 and CD41/CD34 were restored by heparin addition, other lineages not studied in this manuscript are also likely to be either positively or negatively affected.

Our previous analysis of HS epitope expression during hematopoietic commitment in wild-type ESCs highlighted the essential role that particular sulfate epitopes play in this pathway, with specific changes in HS-patterning occurring at the time of hemangioblast specification (19). Therefore, it is likely that sulfation patterning within HS chains is important for correct control of development. Other studies have also highlighted the importance of HS sulfation in embryogenesis. Murine embryos largely devoid of polymer sulfation (NDST1−/−/2−/−) exhibit pre-gastrula lethality similar to Ext1−/− embryos (35). 6-O-Sulfate deficiencies result in lethality at E15.5 (36), with 6-O-sulfation critical for numerous signaling pathways including BMP (37). The observation that the majority of organs in 2-O-sulfate-deficient mice develop normally however suggests 2-O-sulfation is less critical (38). In agreement with this, complete removal of all sulfate groups or N- or 6-O-sulfates alone eliminated the biological activity of heparin, whereas 2-O-sulfate removal had less effect in our system.

A heparin dp12 was the minimum length sequence required for rescue of hematopoiesis in Ext1−/− cells; however,
the extent of rescue could be significantly increased with the addition of dp14 sequences. Subsequent increases in chain length further increased hematopoiesis with a relatively long species (18 saccharides and above) required for restoration of wild-type levels of hematopoietic differentiation in HS-deficient Ext1⁻/⁻ cells. Some growth factors such as FGF require only a relatively short 8-mer oligosaccharide for activity. However other factors such as VEGF require more extended sequences (28, 39). As BMP and Wnt signaling are important for mesodermal differentiation this suggests HS:BMP and HS:Wnt interactions more likely require an extended HS sequence similar to VEGF.

BMP4 is proposed to have a clear role in mesodermal and endothelial specification (15, 16). Consistent with this, Ext1⁻/⁻ cells were unable to support appropriate pSMAD1/5/8 activation, with this re-instated by the addition of soluble heparin. However, we recognize that BMP4 is not the only HS-dependent step in this pathway and that the addition of soluble HS is likely to influence multiple pathways, as indicated by RT-PCR for Wnt/Nodal ligands. Furthermore HS is required for high affinity VEGF/VEGFR/neuropilin complex formation (40, 41) important for hematopoietic commitment from Flk1⁺ hemangioblasts and thus HS is also likely to be required for further differentiation of these lineages.

Adding heparin to wild-type cells is likely to have different effects to the addition of heparin in a HS-deficient system. Addition of very low doses of heparin promoted differentiation of wild-type cells, yet higher concentrations resulted in a significant reduction in hematopoiesis. In wild-type HS competent cells, it is likely that exogenous heparin (in solution) can inhibit differentiation by competing with endogenous cell surface HS for HS-dependent growth factors binding. Therefore at high concentrations exogenous heparin may sequester factors away from the cell surface, disrupting essential endogenous HS-growth factor binding complexes. Our observation that BMP4 signaling was reduced in heparin-treated wild-type EBs supports this hypothesis. A similar effect was seen when concentrations of 10 μg/ml heparin were added to Ext1⁻/⁻ EBs, with the differences in the concentrations of exogenous heparin required highlighting the influence of endogenous HS provision in the differentiation system.

In summary, HS is essential for mesoderm formation, both in the embryo and during in vitro ESC differentiation via EB culture. Using HS-deficient ESCs we have probed the details of this requirement, demonstrating that HS is most likely required for an obligatory phospho-SMAD1/5/8-mediated step, probably downstream of BMP4. Importantly, soluble GAG can be used to recover activity in HS-deficient cells and, at the correct concentration, can increase activity in an HS-compliant system. Signaling and subsequent differentiation requires relatively long heparan sulfate saccharides containing specific GAG sulfation patterns. As well as demonstrating the utility of an HS-deficient system for isolating and highlighting individual signaling events in a highly complex pathway, our study suggests that the addition of heparin saccharides should be considered alongside those of growth factors and cytokines in differentiation protocols.

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