Heparan Sulfate Facilitates FGF and BMP Signaling to Drive Mesoderm Differentiation of Mouse Embryonic Stem Cells

Heparan sulfate (HS) has been implicated in regulating cell fate decisions during differentiation of embryonic stem cells (ESCs) into advanced cell types. However, the necessity and the underlying molecular mechanisms of HS in early cell lineage differentiation are still largely unknown. In this study, we examined the potential of EXT1−/− mouse ESCs (mESCs), that are deficient in HS, to differentiate into primary germ layer cells. We observed that EXT1−/− mESCs lost their differentiation competence and failed to differentiate into Pax6+/neural-precursor cells and mesodermal cells. More detailed analyses highlighted the importance of HS for the induction of Brachyury+ pan-mesoderm as well as normal gene expression associated with the dorso-ventral patterning of mesoderm. Examination of developmental cell signaling revealed that EXT1 ablation diminished FGF and BMP but not Wnt signaling. Furthermore, restoration of FGF and BMP signaling each partially rescued mesoderm differentiation defects. We further show that BMP4 is more prone to degradation in EXT1−/− mESCs culture medium compared with that of wild type cells. Therefore, our data reveal that HS stabilizes BMP ligand and thereby maintains the BMP signaling output required for normal mesoderm differentiation. In summary, our study demonstrates that HS is required for ESC pluripotency, in particular lineage specification into mesoderm through facilitation of FGF and BMP signaling.

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Background: HS has been implicated in regulating ESC differentiation.
Results: Mouse ESCs lacking EXT1 fail to differentiate into mesoderm. Restoration of the FGF and BMP signaling each partially rescued the mesoderm differentiation defect.
Conclusion: HS facilitates FGF and BMP signaling to drive mesoderm differentiation.
Significance: This study shows that HS essentially promotes mesoderm differentiation of mouse ESCs.
regions leading to a highly heterogeneous structure in mature HS (9). The heterogeneity possesses a structural basis for HS to interact with a large variety of protein ligands to modulate a wide range of biological functions (9). In tissues, the HS chains covalently attach to core proteins to form HS proteoglycans (HSPG), such as syndecans, glypican and perlecans, and are present abundantly on the cell surface and in the extracellular matrix where HSPGs interact via HS chains with growth factors, growth factor binding proteins, extracellular proteases, protease inhibitors, chemokines, morphogens, and adhesive proteins to modulate diverse biological functions (9–11). The essential role of HS in development has been manifested by genetic studies targeting HS biosynthetic enzymes (10). For example, mice deficient in Ext1 or Ext2 fail to undergo gastrulation, lack organized mesoderm and extraembryonic tissues, and die during early development (12, 13), illustrating that HS critically regulates early embryogenesis. HS is abundantly expressed by undifferentiated and differentiating ESCs, and becomes increasingly sulfated as ESCs undergo differentiation (14, 15), suggesting that HS may critically modulate ESC differentiation. This presumption has been supported by the examination of NDST1/2−/− or Ext1−/− mouse ESCs (mESCs). NDST1/2−/− mESCs, devoid of N- and 2-O-sulfation, failed to differentiate into endothelial cells, neuronal cells and adipocytes (16–18). ESCs derived from Ext1 deficient mice (conventional Ext1−/− mESCs) lacked HS and could not differentiate into β3-Tubulin-positive neuron, hemangioblasts, hematopoietic, and endothelial cells (15, 19). These studies demonstrate that HS critically participates in ESC differentiation into mid- and terminally differentiated cell types. Very recently, Forsberg et al. observed that NDST1/2−/− mESCs could take the initial step toward differentiation into all three primary germ layers: the endoderm, ectoderm and mesoderm (18). The NDST1/2−/− mESCs express lower sulfated HS and may still allow for partial functionality of the HS chain, therefore the necessity of HS for mESC differentiation into the three germ layers remains unclear. Furthermore, the underlying molecular mechanism has not been explored if HS appeared to be essential for primary germ layer differentiation.

We previously derived a mESC line from conditionally targeted Ext1 mice and then generated Ext1−/− mESC daughter lines by Cre recombinase treatment (20). The Ext1 deletion resulted in a block of lineage commitment due to impaired FGF signaling and the failure to down-regulate pluripotency genes (20). In this study, we bypassed the requirement of HS for lineage commitment by rescuing FGF signaling and then examined the potential of the cells to differentiate into the three primary germ layers. We observed that the Ext1−/− mESCs differentiated into endoderm and Nestin+ neural precursor cells (NPC), but failed to differentiate into Pax6+ NPCs and mesoderm. Examination of mesoderm differentiation-related signaling observed that FGF and BMP, but not Wnt, were disrupted in the Ext1−/− mESCs, and restoration of the FGF and BMP signaling each partially rescued the mesoderm differentiation defect. We also observed that BMP4 was more prone to be degraded in conditioned medium of Ext1−/− ESC differentiation culture than in the wild-type control, revealing that HS stabilizes BMP4 to promote BMP signaling. Therefore our study demonstrates that HS facilitates FGF and BMP signaling to drive mesoderm differentiation of mESCs.

**EXPERIMENTAL PROCEDURES**

**Culture and Differentiation**—Conditionally targeted Ext1 (Ext1+/−) mESC and Ext1−/− daughter cell lines were established as previously described (20). mESCs were maintained in medium consisting of DMEM (Hyclone) supplemented with 10% fetal bovine serum (FBS), 10% KSR (Invitrogen), 1× non-essential amino acids (NEAA), 4 mM l-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.1 mM β-mercaptoethanol, and 1000 units/ml Leukemia Inhibitory Factor (LIF, ESGRO, Chemicon) at 37 °C under 5% CO2. For differentiation, mESCs were cultured in differentiation medium (15% FBS, 4 mM l-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.1 mM β-mercaptoethanol). For FGF signaling rescue experiments, the differentiation medium was supplemented with 200 ng/ml FGF-2 (R&D Systems).

**Quantitative PCR (qRT-PCR) Analysis**—Total RNA was isolated using the RNasy kit (Qiagen) and cDNA was made from 1 μg of total RNA using the SuperScript III First-Strand Synthesis System (Invitrogen). Sybr-Green® RT-PCR was performed as described previously (14). Primers used are listed in supplemental Table S1.

**Immunostaining**—Cells were fixed with 4% PFA for 10 min at room temperature, washed three times with PBS, then incubated for 1 h in blocking buffer (3% BSA, 0.1% Triton X-100 in PBS). The anti-Brachyury antibody (Santa Cruz Biotechnology) was diluted in blocking buffer at 1:100 and applied overnight at 4 °C. Secondary antibodies conjugated to Alexa fluorophores was diluted in blocking buffer at 1:100 and applied for 1 h at room temperature. Cells were washed twice and incubated with DAPI (10 μg/ml) before viewing. Fluorescent images were visualized using a fluorescence microscope (Nikon Eclipse, TE2000-S) with a 20×/0.40 objective at RT and captured using a Qimaging (Retiga 1300i Fast) camera and Qcapture software version 2.90.1.

**FGF Signaling**—mESCs cultured in differentiation medium with or without supplement of FGF-2 at 200 ng/ml were lysed with RIPA buffer containing protease and phosphatase inhibitors (Sigma), and 30 μg of whole cell lysates were resolved on 10% SDS-PAGE gels, transferred onto PVDF membranes, and blotted with anti-phospho-Erk1/2 (Cell Signaling Technology) and anti-Erk1/2 (Cell Signaling Technology) antibodies at 1:1000 (20).

**Wnt Signaling**—Wnt/β-catenin activity was examined using a TCF responsive luciferase reporter vector (TOPflash, M50) containing TCF/LEF binding sites and a control vector (FOPflash, M51) that contains mutant TCF/LEF binding sites (21). Cells were transfected with 0.5 μg of M50/M51 using Fugene (Roche). Transfection of a TK-Revilina construct was used as an internal control. Twenty-four hours after transfection, the medium was changed to serum-free medium containing DMEM + 1X N2 (Gemini Bio-products) + 1× B27 (Gemini Bio-products) with or without 100 ng/ml human Wnt3 (R&D Systems). Six hours later, cells were lysed, and luciferase activity was measured.
**BMP Signaling**—The cells were serum starved for 6 h and then stimulated with 25 ng/ml of BMP-4 (R&D Systems) for 0–60 min, or alternatively, the cells were cultured in differentiation medium for 0–96 h without or with BMP-4 supplemented at 5 ng/ml. Thereafter, cells were lysed with RIPA buffer and 30 μg of whole cell lysates were resolved on 10% SDS-PAGE gels, transferred onto nitrocellulose membranes and blotted with anti-phospho-SMAD1/5/8 (Cell Signaling Technology) and anti-actin (Santa Cruz Biotechnology) antibodies at 1:1000.

**Western Blot Analysis of HSPGs**—mESCs were cultured in ESC medium until confluence and then changed to serum-free media. After an overnight culture, the conditioned media were collected. The collected media were concentrated 10-fold using 0.5 ml 3 kDa cutoff filters (Amicon Ultra Centrifugal Filter, Millipore). A portion of the concentrated media was then subjected to heparin lyase I-III digestion for 1 h at 37 °C. The heparin lyase-treated and untreated media were then resolved on a 6% SDS-polyacrylamide gel, transferred onto PVDF membrane and blotted with anti-HS antibody (10E4, Seikagaku Corporation) and anti-actin (Santa Cruz Biotechnology) antibodies at 1:1000.

Western Blot Analysis of HSPGs—mESCs were cultured in ESC medium until confluence and then changed to serum-free media. After an overnight culture, the conditioned media were collected. The collected media were concentrated 10-fold using 0.5 ml 3 kDa cutoff filters (Amicon Ultra Centrifugal Filter, Millipore). A portion of the concentrated media was then subjected to heparin lyase I-III digestion for 1 h at 37 °C. The heparin lyase-treated and untreated media were then resolved on a 6% SDS-polyacrylamide gel, transferred onto PVDF membrane and blotted with anti-HS antibody (10E4, Seikagaku Corporation) and anti-actin (Santa Cruz Biotechnology) antibodies at 1:1000.

**Quantification of BMP-4**—mESCs were cultured for 48 h in ESC medium, after which medium was changed to DMEM containing 2.5% FBS, 1000 units/ml LIF, 1× NEAA, 0.1 mM β-mercaptoethanol, 4 mM L-glutamine, and 10 ng/ml human BMP-4 with or without heparin (10 μg/ml). After 12, 24, and 36 h, the media were collected, and the amount of human BMP-4 in the culture media was quantified using an enzyme-linked immunosorbent assay kit (Quantikine, R&D Systems). To ensure that the media were collected from wells with equal cell numbers, cells were lysed and protein concentrations were determined.

**RESULTS**

Rescue of FGF Signaling Commits EXT1−/− mESCs to Lineage Differentiation—EXT1 is part of a co-polymerase complex with EXT2 that catalyzes the alternate addition of glucuronic acid and N-acetylglucosamine residues to polymerize the HS chain, which subsequently becomes heavily sulfated. We previously generated EXT1−/− mESC lines by ablating conditionally targeted EXT1 allele with Cre recombinase (20). The EXT1−/− mESCs produce no HS (supplemental Fig. S1) and fail to commit to lineage differentiation due to a defect in FGF signaling (20). The disruption of FGF signaling retains an elevated expression of the pluripotency gene Nanog, thereby preventing cell fate commitment of EXT1−/− mESCs (20). To restore the competence of the EXT1−/− mESCs to commit to lineage differentiation, we strategized to supplement a high dose of FGF-2 (200 ng/ml) to our differentiation medium for the initial 2 days after withdrawal of LIF in order to bypass the requirement of HS in FGF signaling (Fig. 1A) (18, 22). Indeed, the FGF-2 addition restored MAPK signaling and down-regulation of Nanog expression in EXT1−/− mESCs to levels comparable with wild
type cells (Fig. 1, B & C). Consistent with the reduced expression of Nanog, the EXT1<sup>+/−</sup> mESCs exhibited cell morphology alterations that are commonly associated with differentiation commitment of pluripotent cells: compact, dome-shaped colonies began to spread into flat single cell layers and showed that EXT1<sup>+/−</sup> mESCs committed fully to cell differentiation (Fig. 1D). In contrast to the full rescue effect in EXT1<sup>−/−</sup> mESCs, the high FGF-2 supplement did not substantially alter FGF signaling and Nanog expression in EXT1<sup>+/+</sup> ESCs (Fig. 1, B & C). Taken together, these observations show that high FGF-2 supplement successfully committed EXT1<sup>−/−</sup> mESCs to differentiation without inducing substantial changes in the differentiation dynamics of wildtype ESCs. From here onward, we used this differentiation condition as our experimental system to examine the role of HS in early differentiation of mESCs.

EXT1<sup>−/−</sup> mESCs Exhibit Neuroectoderm and Mesoderm Differentiation Defects—To determine the role of HS in early cell differentiation, EXT1<sup>−/−</sup> and EXT1<sup>+/+</sup> mESCs were differentiated in serum-containing medium with FGF2 (200 ng/ml) supplemented for the initial 2 days of the 6-day differentiation scheme (Fig. 1A). The expression of pluripotency genes and early cell lineage-associated genes was determined by qRT-PCR analysis over the time course of differentiation. In agreement with the afore described observations, the expression of pluripotency genes Nanog (Fig. 2A) and Rex1 (data not shown) in EXT1<sup>−/−</sup> mESCs declined over the time course. In contrast, the expressions of endoderm-associated markers (Sox17, Foxa2) were up-regulated (Fig. 2, B & C), indicating that EXT1 ablation stimulated mESC fate to endoderm. The neuroectoderm marker Nestin was induced late and Pax6 was expressed at lower levels altogether in EXT1<sup>−/−</sup> mESCs, showing that HS is required for differentiation into NPCs (Fig. 2, D & E). Strikingly, examination of mesoderm-associated genes observed that the expression of the pan-mesoderm marker Brachyury, and anterior mesoderm marker Goosecoid all failed to become up-regulated (Fig. 2, F–I), indicating that EXT1<sup>−/−</sup> mESCs were unable to differentiate into mesoderm lineage. To determine whether the abnormal mesoderm differentiation of EXT1<sup>−/−</sup> mESCs was truly due to HS deficiency, we supplemented the cell differentiation culture with heparin, a structural analog of HS, and noted that heparin efficiently restored Brachyury expression. We observed that heparin supplement at 10 μg/ml fully restored Brachyury expression in EXT1<sup>−/−</sup> mESCs (Fig. 3A). Consistent with this gene expression profile, immunostaining showed that heparin efficiently restored Brachyury expression in the differentiating EXT1<sup>−/−</sup> mESC populations during the differentiation course (Fig. 3, A & B). These observations demonstrate that HS is required for mESCs to differentiate into mesoderm lineage.
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HS Facilitates FGF Signaling to Promote Mesoderm Differentiation—FGF signaling is known to promote ESCs to differentiate into mesoderm (8, 23–27). To commit the \( \text{EXT1}^{-/-} \) mESCs to cell differentiation, we rescued the FGF signaling only for the first 2 days of differentiation and thereafter the FGF signaling was disrupted again. It is possible that the following FGF signaling disruption may cause the subsequent mesoderm differentiation defect of the \( \text{EXT1}^{-/-} \) mESCs. To test this idea, we continued the FGF supplement through the entire course of differentiation. As shown in Fig. 3A, restoration of FGF signaling partially rescued Brachyury expression in \( \text{EXT1}^{-/-} \) mESCs, showing that HS facilitates FGF signaling to promote mesoderm differentiation. The partial rescue effect also suggested that other signaling pathways required for mesoderm differentiation were also disrupted in \( \text{EXT1}^{-/-} \) mESCs.

Wnt Signaling Alteration Does Not Contribute to the Mesoderm Differentiation Defect of \( \text{EXT1}^{-/-} \) mESCs—Wnts are developmentally regulated molecules that are secreted and expressed by mESCs (28, 29). Canonical Wnt signaling is essentially required for development of mesoderm during mESC differentiation (4–6). HS has been shown to function as a co-receptor, to stabilize and to maintain Wnt gradients to critically modulate Wnt signaling during embryogenesis (30–33), suggesting a possibility that HS may facilitate Wnt signaling to drive mesoderm differentiation of mESCs. To test this hypothesis, we carried out luciferase reporter assay to measure Wnt-reporter activity in both \( \text{EXT1}^{+/-} \) and \( \text{EXT1}^{-/-} \) mESCs. Surprisingly, luciferase activity was consistently higher in \( \text{EXT1}^{-/-} \) ESCs than in \( \text{EXT1}^{+/-} \) ESCs in response to Wnt3 (Fig. 3C), reflecting that HS absence enhanced Wnt signaling during mESC differentiation. The elevated Wnt signaling did not correlate with the mesoderm differentiation defect in \( \text{EXT1}^{-/-} \) mESCs, indicating that the Wnt signaling alteration was not the cause of the mesoderm differentiation defect in \( \text{EXT1}^{-/-} \) mESCs.

HS Facilitates BMP Signaling to Promote Mesoderm Differentiation—BMPs modulate numerous aspects of organogenesis and early embryogenesis by regulating cell proliferation, survival and cell fate (4, 6, 34–36). Smad1/5/8 are direct targets of BMP signaling. During mESC differentiation, BMP/Smad signaling promotes mesoderm formation by acting on the family of \( \text{I} \text{d} \) (\( \text{Inhibitors of DNA binding} \) transcription factors (35). HS has been shown to facilitate BMP and Decapentaplegic (the functional ortholog of mammalian BMP2 and BMP4) signaling to promote organogenesis in mice and \( \text{Drosophila} \) (37–39), suggesting that \( \text{EXT1} \) deficiency may disrupt BMP signaling to lead to the mesoderm differentiation defect in \( \text{EXT1}^{-/-} \) mESCs. To test this hypothesis, we examined activation of Smads as well as induction of \( \text{I} \text{d} \) transcription upon stimulation by BMP-4. We found that phosphorylation of Smad 1/5/8 was
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FIGURE 4. EXT1+/− mESCs show attenuated BMP/Id signaling during differentiation and the attenuation is rescued by heparin. A. Phosphorylation of Smad1/5/8 upon BMP-4 stimulation. mESCs were initially cultured in serum-containing differentiation medium with FGF-2 supplement at 200 ng/ml for 2 days and then were changed to serum-free medium for 6 h. The cells were then stimulated with BMP-4 and phosphorylation of Smad1/5/8 was examined by Western blot analysis. B, phosphorylation of Smad1/5/8 at steady-state differentiation culture. The ESCs were initially cultured in differentiation medium with FGF-2 supplement at 200 ng/ml for 2 days. Following, the cells were cultured in differentiation medium with or without supplement of Noggin and examined for levels of Smad1/5/8 phosphorylation at steady-state culture. Actin levels were used as internal controls. C, Id1 and Id3 expression upon BMP4 stimulation. The mESCs were treated as described in A. Transcript levels of Id1 and Id3 were examined after stimulation of serum-starved differentiating mESCs with BMP-4 in the absence of Noggin. Error bars indicate S.E. generated from triplicates of the same experiment. Statistical analysis was carried out by Student’s t test. 

Reduced in response to BMP-4 in EXT1+/− mESCs compared with BMP-4 in the EXT1+/+ mESC control, and the signaling attenuation was analogous to EXT1+/− mESCs treated with Noggin, a specific BMP signaling inhibitor (Fig. 4, A & B). Furthermore, phosphorylation of Smad1/5/8 at a steady state of differentiation culture was also significantly reduced in EXT1+/− mESCs (Fig. 4B), reflecting that BMP signaling was consistently attenuated during differentiation. Consistent with these observations, the transcript levels of Id1 and Id3 were lower in EXT1+/− and Noggin-treated EXT1+/+ mESCs than in untreated EXT1+/+ mESCs under both examining conditions: shortly after BMP-4 stimulation of serum-starved mESCs, and steady state in serum-free medium containing BMP-4 with or without Noggin addition (Fig. 4, C & D). Heparin addition rescued Smad1/5/8 phosphorylation and partially restored Id1 expression in a dose-dependent manner at concentrations between 1–10 μg/ml (Fig. 4, E & F), supporting our hypothesis that the impaired BMP signaling in EXT1+/− mESCs was due to HS deficiency. Taken together, our results indicate that efficient BMP signaling in mESCs requires HS.

To determine whether EXT1 ablation disrupts BMP signaling to result in the aberrant mesoderm differentiation of EXT1+/− mESCs, we explored whether restoration of BMP signaling would rescue the mesoderm differentiation defect. We first tested to see whether the addition of recombinant BMP-4 could restore BMP signaling/Id1 expression in EXT1+/− mESCs. Indeed, supplementing the differentiation medium with BMP-4 at 2.5–5 ng/ml rescued Id1 expression in EXT1+/− mESCs comparable to that of EXT1+/+ mESCs (Fig. 5A). Next, we examined whether the BMP-4 addition would restore mesoderm differentiation in EXT1+/− mESCs by measuring expression of mesoderm-associated genes that are directly dependent on BMP signaling. We examined two mesoderm-associated genes that have previously been shown to be activated by BMP signaling, Evx1 and Mesp1, and two genes that have been shown to be suppressed by BMP signaling, Hoxb1 and Foxa2, during ESC differentiation (4). Consistent with the previously published results, inhibition of BMP signaling in EXT1+/+ mESCs with Noggin resulted in lower expression of posterior genes Evx1 and Mesp1 and higher expression of anterior genes Hoxb1 and Foxa2 (Fig. 5, B–E). Differentiation of EXT1+/− mESCs in the presence of supplemented BMP-4 at 5 ng/ml elevated Evx1 and Mesp1 expression and suppressed Foxa2 and Hoxb1 expression with mRNA levels approximating
those in \(\text{EXT}1^{+/+}\) mESCs, illustrating directly that the compromised BMP signaling in \(\text{EXT}1^{-/-}\) mESCs leads to their mesoderm differentiation defect (Fig. 5, B–E). In combination with our aforementioned finding that efficient BMP signaling in mESCs requires HS, these results demonstrate that loss of HS leads to aberrant expression of mesodermal genes as a direct result of defective BMP signaling and reveals that HS derives mesoderm differentiation via facilitation of BMP signaling.

**HS Stabilizes BMP-4 to Facilitate BMP Signaling**—Our short-term BMP4-stimulation experiment observed that Samd1/5/8 phosphorylation was significantly attenuated in \(\text{EXT}1^{-/-}\) mESCs compared with \(\text{EXT}1^{+/+}\) mESCs (Fig. 4A), suggesting a co-receptor function for HS in facilitation of BMP signaling, analogous to the well-recognized regulatory mode of HS on FGF signaling (40). This notion was also supported by our transcript analysis showing that mESCs abundantly expressed cell surface HSPGs, including both syndecans and glypicans (Fig. 6A). Meanwhile, we also realized that our mESC differentiation analysis was a long-term experiment that lasted up to 8 days. Under this experimental condition, the stability of BMP4 in culture was a critical requirement for the signaling (41). Extracellular HS has also been suggested to function to stabilize ligand in order to modulate extrinsic signaling (10, 11). Cell surface HSPGs can be shed from the cell surface to extracellular matrix by proteolytic cleavage (42, 43). Meanwhile, our transcript analysis observed that mESCs also highly expressed cell surface HSPGs, including both syndecans and glypicans (Fig. 6A). The presence of extracellular HSPGs in \(\text{EXT}1^{+/+}\) mESCs conditioned medium was confirmed by Western blot analysis probing with anti-HS antibody (Fig. 6B). As predicted, extracellular HSPGs in \(\text{EXT}1^{-/-}\) mESC culture did not carry any HS (Fig. 6B). It is possible that the HS deficiency in conditioned medium may destabilize BMP4 in culture, thereby attenuating BMP signaling in \(\text{EXT}1^{-/-}\) mESCs. This hypothesis was tested initially by examining whether BMP stability was compromised in the \(\text{EXT}1^{-/-}\) mESC culture. Human BMP-4 was supplemented in mESC differentiation culture and the remaining BMP-4 in medium was determined over a time course of 12, 24, and 36 h. We found that BMP-4 levels significantly lower in conditioned \(\text{EXT}1^{-/-}\) mESC culture media than in \(\text{EXT}1^{+/+}\) controls at all the three time points examined (Fig. 6C). Secondly, we tested whether heparin could substitute endogenous HS to restore the stability of BMP-4 in \(\text{EXT}1^{-/-}\) mESC culture by supplementing BMP-4 with heparin. Heparin at 10 \(\mu\)g/ml significantly enhanced the stability of BMP-4 in \(\text{EXT}1^{-/-}\) mESC culture (Fig. 6D). Together, these observations suggest that HS stabilizes BMP-4 to facilitate BMP signaling in mESCs during differentiation.

**DISCUSSION**

The role of HS in mESC differentiation has been examined by targeting various HS biosynthetic genes using both gene knock-out and knockdown approaches (15, 17–20, 29, 44). In this study, we used conditionally targeted \(\text{EXT}1^{-/-}\) mESCs, which produce no HS (20). We observed previously that \(\text{EXT}1^{-/-}\) mESCs sustained self-renewal even in the absence of LIF and demonstrated that HS is required for mESCs to exit from the self-renewal state and to commit to differentiation (20). Other studies have supported this idea and have shown that HS-deficient mESCs remain undifferentiated or fail to commit past the primitive ectoderm stage (15, 17). Here, we induced mESC differentiation by bypassing the requirement for HS in initial FGF-
mediated lineage commitment and thus were able to examine cell fate decisions once mESCs were fully committed. We observed that loss of HS lead to a restricted differentiation potential of ectoderm as manifested in failure or delay to express NPC genes including PAX6 and Nestin, respectively. Similar observations were made by John et al. and Forsberg et al. that used neural differentiation protocols and found similar restrictions in neural development of EXT1/H11002/H11002 and Ndst1/2/H11002 mESCs, respectively (15, 18).

We also observed that EXT1/H11002/H11002 mESCs failed to efficiently give rise to mesodermal precursor cells, revealing the necessity of HS for mesoderm differentiation. In line with our observation, other reports have shown the requirement of HS to generate mesoderm derivatives including hemangioblast and hematopoietic cells (19) and adipocytes (18). Our study suggests that failure to form mesoderm-derivative and terminally differentiated cell types in HS mutant cells may arise from an early block in transitioning from stem cell stage to mesodermal precursors after the cells committed to cell differentiation.

Several extrinsic signaling pathways, including FGF, BMP, and Wnt, function to promote mesoderm differentiation of mESCs (5, 6, 8, 26, 27, 45–47). We observed that EXT1 ablation attenuated FGF and BMP, but not the Wnt signaling. The attenuation of FGF and BMP signaling correlated positively with the disrupted mesoderm differentiation phenotype, and more directly, we observed that restoration of FGF and BMP4 signaling each partially rescued the mesoderm differentiation defect, demonstrating that disruption of FGF and BMP4 signaling were the major reason for the aberrant mesoderm differentiation of EXT1/H11002/H11002 mESCs. These observations led us to conclude that HS facilitates FGF and BMP signaling to drive mesoderm differentiation of mESCs.

It is well-known that endogenous HS functions as a co-receptor to facilitate FGF signaling, but the regulatory role of endogenous HS on BMP signaling appears to be more complex and depends on cell/tissue context. Our study demonstrated that HS facilitates BMP signaling in the context of mESCs. This observation is in agreement with previous reports showing that HS depletion by heparinase treatment or by knockdown of the PAPST, the transporter that transports PAPS from cytosol to Golgi for sulfation modification at HS biosynthesis, both attenuated BMP signaling in mESCs (29, 44). Interestingly, Manton et al. observed that HS depletion by heparinase treatment enhanced BMP signaling in human mesenchymal stem cells during differentiation into osteoblasts (48), indicating that HS functions to inhibit BMP signaling in the context of human mesenchymal stem cells. The positive and negative regulatory roles of HS in BMP signaling have been also observed during embryogenesis in EXT1- or Ndst1-deficient mice, respectively (37, 49). Several mechanisms have been suggested for HS to
modulate BMP signaling, including co-reception, stabilization and maintaining gradient of the ligand, and mediation of BMP internalization (39, 50). In our study, BMP signaling was attenuated in \textit{EXT1}^{-/-} mESCs upon BMP stimulation in short-term experiments, suggesting that HS may function as a co-receptor for BMP signaling. We also observed that BMP is more prone to degradation in \textit{EXT1}^{-/-} mESC culture and that this degradation could be inhibited by heparin. This observation revealed that HS stabilizes BMP during mESC differentiation. Therefore, our observations suggest HS functions through both co-receptor and ligand stabilization modes to facilitate BMP signaling during mESC differentiation.

We observed that Wnt signaling was up-regulated in differentiating \textit{EXT1}^{-/-} mESCs, indicating that HS negatively modulates Wnt signaling in the context of differentiating mESCs. A similar effect was also observed by Manton et al. during differentiation of human mesenchymal stem cells into osteoblasts (48). Since the up-regulation of Wnt signaling did not correlate with the disrupted mesoderm differentiation phenotype of \textit{EXT1}^{-/-} mESCs, we postulated that the Wnt signaling alteration was not the molecular mechanism underlying the mesoderm differentiation abnormality. Previous studies by Sasaki et al. also examined the effect of HS deficiency on Wnt signaling in mESCs, and observed that knockdown of \textit{EXT1} or PAPSTs, both attenuated Wnt signaling, showing that HS positively regulates Wnt signaling in mESCs (29, 44). Cell surface HS has been suggested to function as a co-receptor to facilitate Wnt signaling as implicated in the \textit{EXT1}-KD and PAPST-KD studies (29, 44) or in a “catch” mode to negatively modulate Wnt signaling (51). We tend to believe that cell surface HS functions in both modes at the same time to finely modulate Wnt signaling. However the apparent effect is determined by which mode plays a dominant role in the specific experimental system. The up-regulation of Wnt signaling in our \textit{EXT1}^{-/-} mESCs indicates that HS functions to negatively modulate Wnt signaling and suggests that the “catch” mode plays a dominant role in the context of differentiating mESCs. Based on our belief, we also tentatively postulate that in the cases of \textit{EXT1}-KD and PAPST-KD mESCs, the short or undersulfated HS carried by the cells might selectively lose its co-receptor function, thereby enhancing the already dominant “catch” function mode to lead to down-regulation of Wnt signaling in \textit{EXT1}-KD or PAPST-KD mESCs.

During embryogenesis, development is controlled by the concerted action of different cell signaling pathways that determine the fate of any given cell. Pluripotent ESCs respond to growth factors and morphogens to activate developmental signaling in much the same fashion as the pluripotent cells of the epiblast, therefore representing a suitable system to study the complexities of early mammalian development (2). \textit{EXT1}^{-/-} mice have been observed to lack organized mesoderm, but the related developmental signaling defect has not been known (12). In this study, the differentiation of \textit{EXT1}^{-/-} mESCs recapitulated the aberrant mesoderm phenotype of \textit{EXT}^{-/-} embryos, suggesting that HS may function through a similar molecular mechanism, that is the FGF and BMP signaling, to modulate mesoderm development during early embryogenesis. The BMP receptor and FGF receptor knock-out mice both exhibited failure in gastrulation and mesoderm formation (25, 52), phenocopying the developmental defect of \textit{EXT1}^{-/-} mice. Therefore, these observations are in line with our \textit{EXT1}^{-/-} mESC study and support that HS deficiency may disrupt BMP and FGF signaling to lead to the mesoderm development defect of the \textit{EXT1}^{-/-} mice.

In conclusion, our results demonstrate that HS directs lineage fate decisions of mESCs into mesodermal precursors through modulation of both FGF and BMP signaling. We show that HS is required for the transitioning of mESCs through a Brachyury+ pan-mesodermal precursor stage as well as for dorso-ventral patterning of ESC via facilitation of FGF and BMP signaling, respectively. In this respect we have also elucidated that HS modulates BMP signaling by enhancing ligand stability. Further studies that examine the fine structure requirements of HS for developmental signaling and their effects on fate decision during ESC differentiation will provide us with means to generate specific cell types for cell replacement therapies.

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**REFERENCES**

1. Evans, M. J., and Kaufman, M. H. (1981) Establishment in culture of pluripotential cells from mouse embryos. Nature **292**, 154–156.
2. Keller, G. (2005) Embryonic stem cell differentiation: emergence of a new era in biology and medicine. Genes Dev. **19**, 1129–1155.
3. Wilson, A., and Trump, A. (2006) Bone-marrow haematopoietic-stem-cell niches. Nat. Rev. Immunol. **6**, 93–106.
4. Lengerke, C., Schmitt, S., Bowman, T. V., Jang, I. H., Maouche-Chretien, L., McKinney-Freeman, S., Davidson, A. J., Hammerschmidt, M., Rentzsch, F., Green, J. B., Zon, L. I., and Daley, G. Q. (2008) BMP and Wnt specify hematopoietic fate by activation of the Cdx-Hox pathway. Cell Stem. Cell **2**, 72–82.
5. Lindsley, R. C., Gill, J. G., Kyba, M., Murphy, T. L., and Murphy, K. M. (2006) Canonical Wnt signaling is required for development of embryonic stem-cell-derived mesoderm. Development **133**, 3787–3796.
6. Nostro, M. C., Cheng, X., Keller, G. M., and Gadue, P. (2008) Wnt, activin, and BMP signaling regulate distinct stages in the developmental pathway from embryonic stem cells to blood. Cell Stem. Cell **2**, 60–71.
7. Ying, Q. L., and Smith, A. G. (2003) Defined conditions for neural commitment and differentiation. Methods Enzymol. **365**, 327–341.
8. Willems, E., and Leyns, L. (2008) Patterning of mouse embryonic stem cell-derived pan-mesoderm by Activin A/Nodal and Bmp4 signaling requires Fibroblast Growth Factor activity. Differentiation **76**, 745–759.
9. Esko, J. D., and Selleck, S. B. (2002) Order out of chaos: assembly of ligand binding sites in heparan sulfate. Annu. Rev. Biochem. **71**, 435–471.
10. Bishop, J. R., Schuksz, M., and Esko, J. D. (2007) Heparan sulfate proteoglycans fine-tune mammalian physiology. Nature **446**, 1030–1037.
11. Bernfield, M., Götte, M., Park, P. W., Reizes, O., Fitzgerald, M. L., Lincum, J., and Zako, M. (1999) Functions of cell surface heparan sulfate proteoglycans. Annu. Rev. Biochem. **68**, 729–777.
12. Lin, X., Wei, G., Shi, Z., Dryer, L., Esko, J. D., Wells, D. E., and Matzuk, M. M. (2000) Disruption of gastrulation and heparan sulfate biosynthesis in \textit{EXT1}-deficient mice. Dev. Biol. **224**, 299–311.
13. Stickens, D., Zak, B. M., Rougier, N., Esko, J. D., and Werb, Z. (2005) Mice deficient in Ext2 lack heparan sulfate and develop exostoses. Development **132**, 5055–5068.
14. Nairn, A. V., Kinoshita-Toyoda, A., Toyoda, H., Xie, J., Harris, K., Dalton, S., Kulik, M., Pierce, J. M., Toida, T., Moremen, K. W., and Linhardt, R. J.
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(2007) Glycomics of proteoglycan biosynthesis in murine embryonic stem cell differentiation. J. Proteome Res. 6, 4374–4387

15. Johnson, C. E., Crawford, B. E., Stavridis, M., Ten Dam, G., Wat, A. L., Rushton, G., Ward, C. M., Wilson, V., van Kuppevelt, T. H., Esko, I. D., Smith, A., Gallagher, J. T., and Merry, C. L. (2007) Essential alterations of heparan sulfate during the differentiation of embryonic stem cells to Sox1-enhanced green fluorescent protein-expressing neural progenitor cells. Stem Cells 25, 1913–1923

16. Jakobsson, L., Kreuger, J., Holmborn, K., Lundin, L., Eriksson, I., Kjellén, L., and Claesson-Welsh, L. (2006) Heparan sulfate in trans potentiates VEGF-Mediated angiogenesis. Dev. Cell 10, 625–634

17. Lanner, F., Lee, K. L., Sohl, M., Holmborn, K., Yang, H., Wilbertz, J., Poellinger, L., Rossant, J., and Farnebo, F. (2010) Heparan sulfation-dependent fibroblast growth factor signaling maintains embryonic stem cells primed for differentiation in a heterogeneous state. Stem Cells 28, 191–200

18. Forsberg, M., Holmborn, K., Kundu, S., Dagalv, A., Kjellen, L., and Forsberg-Nilsson, K. (2012) Under-sulfation of heparan sulfate restricts the differentiation potential of mouse embryonic stem cells J. Biol. Chem., in press

19. Holley, R. J., Pickford, C. E., Rushton, G., Lacaud, G., Gallagher, J. T., Kouskoff, V., and Merry, C. L. (2011) Influencing hematopoietic differentiation of mouse embryonic stem cells using soluble heparin and heparan sulfate saccharides. J. Biol. Chem. 286, 6241–6252

20. Kraushaar, D. C., Yamaguchi, Y., and Wang, L. (2010) Heparan sulfate is required for embryonic stem cells to exit from self-renewal. J. Biol. Chem. 285, 5907–5916

21. Bibichele, T. L., and Moon, R. T. (2008) Assaying β-catenin/TCF transcription with beta-catenin/TCF transcription-based reporter constructs. Methods Mol. Biol. 468, 99–110

22. Padera, R., Venkataraman, G., Berry, D., Godavarti, R., and Sasisekharan, R., Ikenaka, K., and Nishihara, S. (2008) Heparan sulfate regulates self-renewal and pluripotency of mouse embryonic stem cells. Biochem. Soc. Trans. 36, 45–49

23. Zheng, Z., de Jongh, R. U., Rathjen, P. D., and Rathjen, J. (2007) Essential alterations of heparan sulfate during the differentiation of embryonic stem cells to Sox1-primed for differentiation in a heterogeneous state. Stem Cells 28, 191–200

24. Chambers, I., Colby, D., Robertson, M., Nichols, J., Lee, S., Tweedie, S., and Smith, A. G. (2003) Functional expression cloning of Nanog, a pluripotency factor dimerization, activation, and cell proliferation. Cell 79, 1015–1024

25. Kuo, W. J., Digman, M. A., and Lander, A. D. (2010) Heparan sulfate acts as a bone morphogenetic protein coreceptor by facilitating ligand-induced receptor hetero-oligomerization. Mol. Biol. Cell 21, 4028–4041

26. Fitzgerald, M. L., Wang, Z., Park, P. W., Murphy, G., and Bernfield, M. (2000) Shedding of syndecan-1 and -4 ectodomains is regulated by multiple signaling pathways and mediated by a TIMP-3-sensitive metalloproteinase. J. Cell Biol. 148, 811–824

27. Subramanian, S. V., Fitzgerald, M. L., and Bernfield, M. (1997) Regulated shedding of syndecan-1 and -4 ectodomains by thrombin and growth factor receptor activation. J. Biol. Chem. 272, 14713–14720

28. Sasaki, N., Hirano, T., Ichimiya, T., Wako, M., Hirano, K., Kinoshita-Toyoda, A., Toyoda, H., Suda, Y., and Nishihara, S. (2009) The 3-phosphosildenafil-5-phosphosulfate transporters, PAPST1 and 2, contribute to the maintenance and differentiation of mouse embryonic stem cells. PLoS One 4, e8262

29. Boyd, N. L., Dhara, S. K., Rekaya, R., Godfrey, E. A., Hasneen, K., Rao, R. R., West, F. D., 3rd, Gerwe, B. A., and Stice, S. L. (2007) BMP4 promotes formation of primitive vascular networks in human embryonic stem cell-derived embryoid bodies Exp. Biol. Med. 232, 833–843

30. Miura, S., Davis, S., Klingensmith, J., and Mishina, Y. (2006) BMP signaling in the epiblast is required for proper recruitment of the prospective paraxial mesoderm and development of the somites. Development 133, 3767–3775

31. Gadue, P., Huber, T. L., Paddison, P. J., and Keller, G. M. (2006) Wnt and TGF-β signaling are required for the induction of an in vitro model of primitive streak formation using embryonic stem cells. Proc. Natl. Acad. Sci. U.S.A. 103, 16806–16811

32. Manton, K. J., Leong, D. F., Cool, S. M., and Nurcombe, V. (2007) Disruption of heparan and chondroitin sulfate signaling enhances mesenchymal stem cell-derived osteogenic differentiation via bone morphogenetic protein signaling pathways. Stem Cells 25, 2845–2854

33. Hu, Z., Wang, C., Xiao, Y., Sheng, N., Chen, Y., Xu, Y., Zhang, L., Mo, W., Jing, N., and Hu, G. (2009) Ndst1-dependent heparan sulfate regulates BMP signaling and internalization in lung development. J. Cell Sci. 122, 1145–1154

34. Dejima, K., Kanai, M. I., Aikiyama, T., Levings, D. C., and Nakato, H. (2011) Novel contact-dependent bone morphogenetic protein (BMP) signaling mediated by heparan sulfate proteoglycans. J. Biol. Chem. 286, 17103–17111

35. Ai, X., Do, A. T., Lozynska, O., Kusche-Gullberg, M., Lindahl, U., and Emerson, C. P., Jr. (2003) QSim1 remodels the 6-O sulfation states of cell surface heparan sulfate proteoglycans to Wnt signaling. J. Cell Biol. 162, 341–351

36. Mishina, Y., Suzuki, A., Ueno, N., and Behringer, R. R. (1995) Bmpr encodes a type I bone morphogenetic protein receptor that is essential for gastrulation during mouse embryogenesis. Genes Dev. 9, 3027–3037