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Heparan Sulfate Is Required for Embryonic Stem Cells to Exit from Self-renewal*

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Pluripotent embryonic stem cells (ESCs) must select between alternative fates of self-renewal and lineage commitment at each division during continuous proliferation. Heparan sulfate (HS) is a highly sulfated polysaccharide and is present abundantly on the ESC surface. In this study, we investigated the role of HS in ESC self-renewal by examining *Ext1*$^{-/-}$ ESCs that are deficient in HS. We found that *Ext1*$^{-/-}$ ESCs retained their self-renewal potential but failed to transit from self-renewal to differentiation upon removal of leukemia inhibitory factor. Furthermore, we found that the aberrant cell fate commitment is caused by defects in fibroblast growth factor signaling, which directly retained high expression of the pluripotency gene *Nanog* in *Ext1*$^{-/-}$ ESCs. Therefore, our studies identified and defined HS as a novel factor that controls ESC fate commitment and also delineates that HS facilitates fibroblast growth factor signaling, which, in turn, inhibits *Nanog* expression and commits ESCs to lineage differentiation.

Embryonic stem cells (ESCs)[2] are derived from the inner cell mass of the preimplantation blastocyst and can differentiate into numerous cell types representative of all three germ layers of the embryo, a property that is defined as pluripotency (1, 2). ESCs retain pluripotency through a process of self-renewal, which allows ESCs to proliferate infinitely as undifferentiated entities. These properties make ESCs a unique system to study early embryonic development and cell fate decisions and provide us with a promising source for cell replacement therapies (3–5). The regulatory network and molecular requirements for the maintenance of self-renewal have been under intense investigation and are now increasingly defined. However, the mechanisms by which ESCs exit the self-renewing state and initiate differentiation are still poorly understood. For example, recent reports suggest that extrinsic signaling of fibroblast growth factors (FGFs) and intracellular factors, including the chromatin-associated protein UTF1 (undifferentiated embryonic cell transcription factor 1) and the nucleosome remodeling complex, NuRD, are instrumental in triggering the exit of ESCs from their self-renewal program and to commit to differentiation (6–8).

Heparan sulfate (HS) is a highly sulfated glycosaminoglycan molecule and is biosynthesized in the Golgi apparatus of cells (9, 10). The copolymerases EXT1 and EXT2 initiate HS biosynthesis by alternately adding glucuronic acid and *N*-acetylglucosamine residues to form HS precursors. Following chain elongation, *N*-deacetylase/*N*-sulfotransferases act on discrete regions of the HS precursors, replacing *N*-acetyl groups with *N*-sulfate and creating appropriate substrates for further modification reactions, including epimerization and *O*-sulfation. The modification reactions are incomplete and result in mature HS that is structurally highly heterogeneous and possesses the potential to interact with a large variety of protein ligands. In tissues, the HS chains covalently attach to core proteins to form HS proteoglycans, such as syndecans, glypicans, and perlecan, and are present abundantly on the cell surface and in the extracellular matrix, where they interact with growth factors, growth factor-binding proteins, extracellular proteases, protease inhibitors, chemokines, morphogens, and adhesive proteins to modulate diverse biological functions (10).

The interactions of HS with growth factors and morphogens, including FGFs, bone morphogenetic proteins (BMPs), Wnts, and Hh (Hedgehog), have been known to play important roles in *Drosophila* development and embryogenesis (11–14). In the context of these signaling molecules, HS is implicated in facilitating receptor-ligand interactions and maintaining morphogen gradients (13–16). Conventional knock-out of HS biosynthetic gene *Ext1* or *Ext2* in mice results in early developmental defects, including the failure to fully develop extraembryonic structures and the lack of any organized mesoderm (17, 18), illustrating that HS critically regulates mammalian embryogenesis and coordinates cell differentiation events.

HS is abundant on the cell surface of undifferentiated and differentiating ESCs (19–23). Recent reports have demonstrated that the HS structure changes as ESCs undergo differentiation and that specific HS epitopes appear on subpopulations of differentiated ESCs, suggesting that HS regulates ESC differentiation and cell lineage development (21, 22, 24). This presumption has been supported by the examination of ESCs lacking *Ndst1/2* (*N*-deacetylase/*N*-sulfotransferase 1/2) or *Ext1*. *Ndst1/2* null ESCs, which are devoid of *N*-sulfation and 2-*O*-sulfation, fail to differentiate into endothelial cells (19, 25). ESCs deficient in *Ext1* do not produce any HS and could not be

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[2] The abbreviations used are: ESC, embryonic stem cell; FGF, fibroblast growth factor; FGFR, FGF receptor; HS, heparan sulfate; BMP, bone morphogenetic protein; EB, embryoid body; LIF, leukemia inhibitory factor; AP, alkaline phosphatase; RT, reverse transcription; PBS, phosphate-buffered saline; PD, PD173074; MAPK, mitogen-activated protein kinase.

induced to differentiate into neuronal cell types (21). These studies illustrate that HS is essential for ESC differentiation into multiple cell lineages. However, the role of HS in ESC self-renewal and cell fate commitment is not known.

In this study, we examined the roles of HS in self-renewal and cell fate commitment of mouse ESCs. We observed that, although HS is not required for the maintenance of ESC self-renewal, it promotes the transition of ESCs from self-renewal to lineage commitment via facilitation of FGF signaling and retention of high *Nanog* expression.

EXPERIMENTAL PROCEDURES

Isolation of ESC Lines—The generation of the conditional *Ext1* allele ($Ext1^{flox}$) and $Ext1^{flox/flox}$ mice was reported previously (26). $Ext1^{flox/flox}$ ESCs were derived from $Ext1^{flox/flox}$ blastocysts according to a standard protocol (27). To obtain *Ext1* null ($Ext1^{-/-}$) daughter ESC lines, $Ext1^{flox/flox}$ ESCs were transfected with pBS513 EF1 α Cre (Addgene) using Lipofectamine 2000, followed by single cell cloning. The alleles of $Ext1^{flox}$ *and* $Ext1^{-}$ were identified by PCR analysis using the following primers. The $Ext1^{floxed}$ allele was amplified using the following primers: 5′-GGAGTGTGGATGAGTTGAAG-3′ (forward) and 5′-CAACACTTTCAGCTCCAGTC-3′ (reverse). The $Ext1^{-}$ allele was amplified using the following primer pair: 5′-GGAGTGTGGATGAGTTGAAG-3 and 5′-GAGAACAGGTACCCATGTTC-3′. Cycling parameters for PCR were 95 °C for 15 min, 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min repeated for 40 cycles and then 72 °C for 10 min.

Culture and Differentiation—ESCs were maintained in medium consisting of Dulbecco's modified Eagle's medium (Hyclone) supplemented with 10% fetal bovine serum, 10% knockout serum replacement (Invitrogen), L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.1 mM β-mercaptoethanol at 37 °C under 5% CO_2, supplemented with 1000 units/ml leukemia inhibitory factor (LIF) (ESGRO, Chemicon). For differentiation into embryoid bodies (EBs), ESCs were trypsinized and transferred into bacterial dishes at 5×10^4 cells/ml in 15% fetal bovine serum without the addition of LIF, and medium was changed every second day. Differentiation in adherent serum-containing culture was achieved in 10% fetal bovine serum, 10% knockout serum replacement without LIF. Serum-free culture was performed in Dulbecco's modified Eagle's medium supplemented with 1× N2 (Gemini), 1× B27 (Gemini), 50 μg/ml bovine serum albumin (Sigma), and 10 ng/ml FGF-4.

Self-renewal Assay—ES cells were seeded at clonal density and cultured for 5 days in ES medium with LIF. Cells were washed, fixed, and tested for alkaline phosphatase (AP) activity using an AP kit (Millipore). One hundred colonies were scored, and the percentage of AP-positive colonies was calculated. Dome-shaped colonies with tightly packed AP-positive cells were considered undifferentiated. Colonies with a mixture of stained and unstained colonies and with flattened and non-uniform morphology were considered differentiated.

Flow Cytometry—For detection of cell surface HS, ESCs were incubated with an anti-HS antibody (H10E4, Seikagaku) at 1:500 and labeled with a secondary antibody (anti-mouse IgM-fluorescein isothiocyanate) before fluorescence-activated cell sorting analysis. FGF cell surface binding was performed as described previously (28). Briefly, ESCs were detached with 2 mM EDTA in PBS for 10 min at room temperature. Cells were incubated with 0.6 μg/ml biotinylated FGF-2 in buffer (0.5% bovine serum albumin, 2 mM EDTA in PBS) for 1 h on ice. Bound FGF-2 was detected by flow cytometry with avidin-fluorescein isothiocyanate (1:1000; R&D Systems).

TABLE 1
Primers for semiquantitative PCR
Primer sequences are shown 5′ to 3′.

Gene	Forward primer	Reverse primer
Oct-4	GGCGTTCTCTTTGGAAAGGTGTTC	CTCGAACCACATCCTTCTCT
Nanog	GCGGACTGTGTGTTCTCTGAGGC	TTCCAGATCCGTTCACCAGATAG
Gata-4	CGAGATGGGACGGGACACT	CTCACCCTCGGCCATTACGA
α-Fetoprotein (*Afp*)	TGCAGAAACACATCGAGGAGAG	GCTTCACCAGGTTAAGAGAAGCT
Brachyury	AACTTTCCTCCATGTGCTGAGAC	TGACTTCCCAACACAAAAAGCT
MixL1	ACTTTCCAGCTCTTTCAAGAGCC	ATTGTGTACTCCCCAACTTTCCC
Flk-1	AAGGAACTAGAATGCGGGCT	ACTCCCTGCTTTTACTGGGC
Foxa1	TGGTCACTGGGGACAAGGGAA	GCAACAACAGCAATAGAGAAC
Ext1	TCCCTGGAGGATTGTTCGTC	TAGCAGCTCCTGTGAACAC
β-Actin	CCTAAGGCCAACCGTGAAAAG	TCTTCATGGTGCTAGGAGC

Immunofluorescence—Cells were fixed in 4% paraformaldehyde for 10 min at room temperature, washed three times with PBS, and then incubated for 1 h in blocking buffer (2% goat serum, 0.1% Triton X-100 in PBS). The anti-OCT-4 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was diluted in blocking buffer at 1:100 and applied for 1 h at room temperature or overnight at 4 °C. Secondary antibodies conjugated to Alexa fluorophores (Molecular Probes) were diluted at 1:500 in blocking buffer and applied for 1 h at room temperature. Cells were washed twice and incubated with 4′,6-diamidino-2-phenylindole (10 μg/ml) before viewing. Fluorescent images were visualized using a fluorescence microscope (Nikon Eclipse, TE2000-S) with ×20/0.40 and ×40/0.60 objectives at room temperature and captured using a Qimaging (Retiga 1300i Fast) camera and Qcapture version 2.90.1 software. Fluorescent confocal images were visualized using an Olympus FV1000 laser-scanning confocal microscope with oil objectives (×40/1.35) at room temperature and captured using Fluoview acquisition software. All images were prepared using Photoshop 8.0 (Adobe).

Immunoblotting—After 6 h of serum starvation, ESCs were stimulated with 5 ng/ml FGF2 for 0–60 min. Thereafter, cells were lysed with radioimmune precipitation buffer, and 30 μg of whole cell lysates were resolved on 10% SDS-polyacrylamide gels, transferred onto nitrocellulose membranes, and blotted with anti-phospho-ERK1/2 (Cell Signaling Technology) and anti-ERK1/2 (Cell Signaling Technology) antibodies at 1:1000.

PCR Analysis—Total RNA was isolated using the RNeasy kit (Qiagen), and cDNA was made from 1 μg of total RNA using the SuperScript III first strand synthesis system (Invitrogen). Semiquantitative PCR was performed with the primers listed in Table 1. SYBR Green® RT-PCR was performed as described previously (22) to detect expression of *Ext1*, *Ext2*, *Nanog*, *Brachyury*, *Wnt3*, *Nestin*, and *Gata-6* with the following primers: *Ext1* forward, 5′-gaggagtcttgctcctcac-3′; *Ext1* reverse, 5′-tgtcacagcgagaatccttg-3′; *Ext2* forward, 5′-acgtcaccctgttctccatc-3′; *Ext2* reverse, 5′-caatggagtgaggccagaac-3′; *Nanog* forward, 5′-ggactttctgcagccttacg-3′; *Nanog* reverse, 5′-gcttccaaattcacctccaa-3′; *Brachyury* forward, 5′-cctgcacattacacaccactg-3′; *Brachyury* reverse, 5′-atcggagaaccagaagacga-3′; *Wnt3*

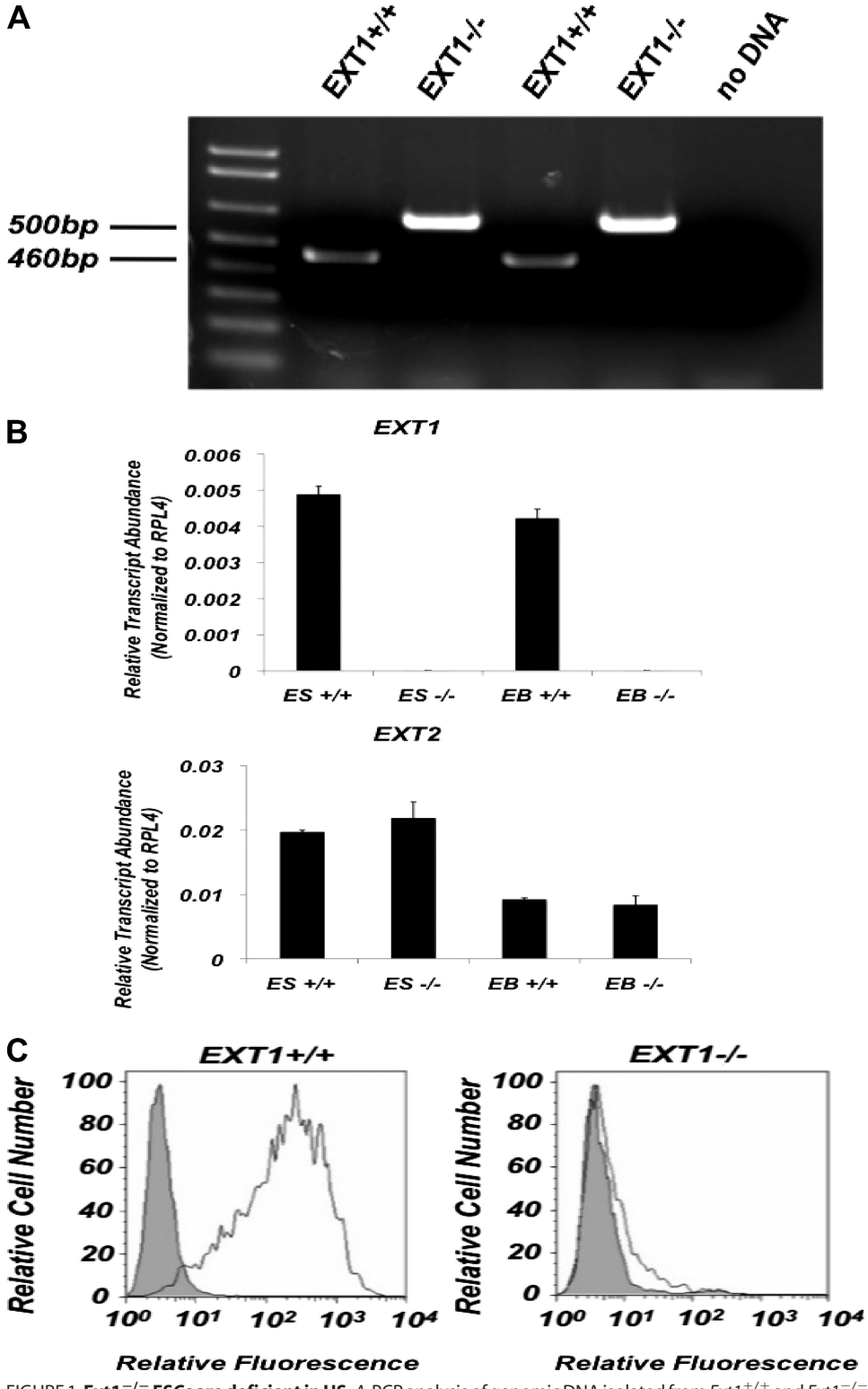


FIGURE 1. **Ext1$^{-/-}$ ESCs are deficient in HS.** *A*, PCR analysis of genomic DNA isolated from *Ext1*$^{+/+}$ and *Ext1*$^{-/-}$ ESC clones. Amplification of the *Ext1*^{flox} allele yields a 460-bp product. Amplification of the *Ext1*$^{-}$ allele yields a 500-bp product. *B*, RT-PCR analysis of *Ext1* and *Ext2* transcripts from *Ext1*$^{+/+}$ and *Ext1*$^{-/-}$ ESCs (*ES*) and EBs. *C*, detection of HS by flow cytometry using an anti-HS antibody (H10E4). Background controls are represented by *shaded peaks*.

forward, ggtggagaaacaccgtgagt; *Wnt3* reverse, tgaagagcgcgtacttagcc; *Nestin* forward, 5′-cgtctacaggcagcgctaac-3′; *Nestin* reverse, 5′-atctgtcaagatcgggatgg-3′; *Gata-6* forward, 5′-caggtcaagacggcctctac-3′; *Gata-6* reverse, 5′-cacggaggatgtgacttcg-3′; glyceraldehyde-3-phosphate dehydrogenase forward, 5′-acgggaagctcactggcatg-3′; glyceraldehyde-3-phosphate dehydrogenase reverse, 5′-ccaggcggcacgtcagatc-3′.

HS Isolation—The HS isolation was carried out as we reported previously (28, 29). In brief, ESCs were washed with ice-cold PBS followed by lysis for 20 min in 0.1 M NaOH. The cell lysate was adjusted to pH 7.0 and then loaded onto a DEAE-Sephacel column (Bio-Rad) that had been equilibrated with equilibration buffer (20 mM sodium acetate, pH 6.0, 0.25 M NaCl). After washing with the equilibration buffer, glycosaminoglycans were eluted with elution buffer (20 mM sodium acetate, pH 6.0, 1 N NaCl). The samples were desalted in water on PD10 columns (Amersham Biosciences), followed by lyophilization, and glycosaminoglycans were subjected to digestion with 20 milliunits of chondroitinase ABC (Sigma) and reduction with $NaBH_4$ overnight. Following an additional desalting step, total HS was quantified using 1,9-dimethylmethylene blue (30).

RESULTS

Ext1$^{-/-}$ *ESCs Are Deficient in HS*—The enzyme EXT1 functions to initiate HS biosynthesis by polymerizing the HS precursor structure. To study the role of HS in ESCs, mouse ESC lines were derived from conditionally targeted *Ext1* (*Ext1*$^{flox/flox}$) mice following standard procedures (26, 27). The *Ext1* null (*Ext1*$^{-/-}$) ESC lines were generated by transfecting *Ext1*$^{flox/flox}$ ESC lines with Cre recombinase, followed by single cell cloning and PCR determination of *Ext1* ablation (Fig. 1*A*). The PCR-identified *Ext1*$^{-/-}$ ESCs were deficient in *Ext1* transcripts but retained normal levels of *Ext2* mRNA (Fig. 1*B*). To determine whether *Ext1* ablation eliminated HS biosynthesis, *Ext1*$^{flox/flox}$ (wild type control, designated as *Ext1*$^{+/+}$) and *Ext1*$^{-/-}$ ESCs were incubated with an anti-HS antibody (H10E4), and HS chains were quantified by flow cytometry. *Ext1*$^{+/+}$ ESCs expressed HS abundantly on their cell surfaces. In contrast, *Ext1*$^{-/-}$ ESCs did not display any HS expression (Fig. 1*C*),

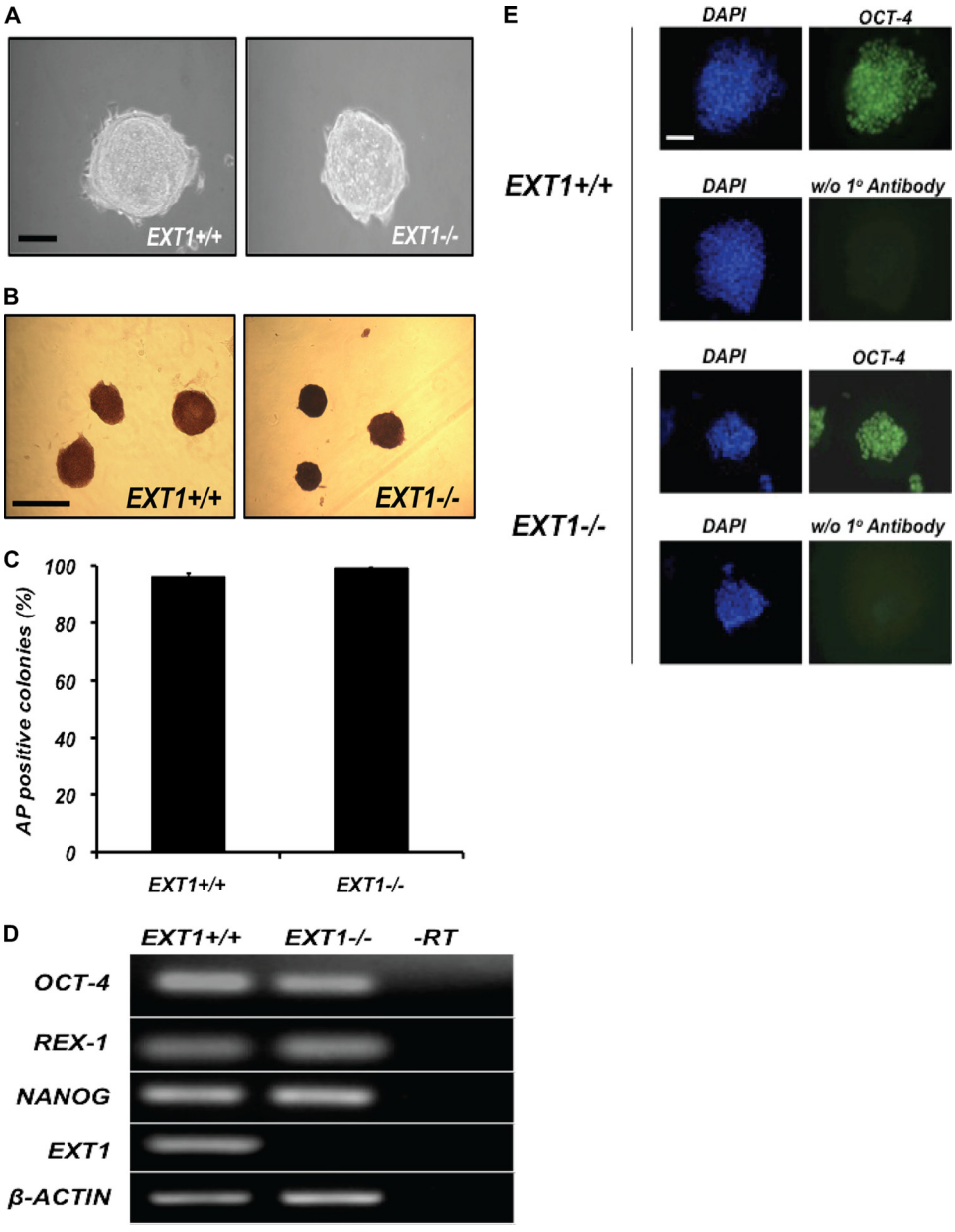


FIGURE 2. **Ext1$^{-/-}$ ESCs can be maintained in the self-renewing state.** *A*, *Ext1*$^{+/+}$ and *Ext1*$^{-/-}$ ESCs were examined for their colony morphology after 20 days (5 passages) in feeder-free conditions in the presence of LIF. *Bar*, 300 μm. *B*, *Ext1*$^{+/+}$ and *Ext1*$^{-/-}$ ESCs were stained for AP activity after 20 days of culture in the presence of LIF. *Bar*, 200 μm. *C*, ESCs were cultured for 15 days and then plated out at clonal density. The percentage of AP-positive colonies was quantitated. *Error bars* indicate the S.E. generated from triplicates of the same experiment, which is representative of at least three independent experiments. *D*, semiquantitative PCR of pluripotency genes. RNA was extracted from ESCs cultured for 20 days in feeder-free conditions. *E*, immunofluorescence of OCT-4 expression in colonies formed after 20 days in culture in feeder-free conditions in the presence of LIF. *Bar*, 150 μm. *DAPI*, 4′,6-diamidino-2-phenylindole.

showing directly that *Ext1* ablation completely disrupts HS biosynthesis in ESCs.

HS Is Not Required for the Maintenance of ESC Self-renewal—Growth factors, including BMP-4 and Wnt, are promoters of ESC self-renewal and are modulated by HS during invertebrate and vertebrate embryogenesis, suggesting that HS might similarly modulate BMP-4 and Wnt signaling to govern ESC self-renewal. To test this idea, we examined *Ext1*$^{-/-}$ ESCs under long term feeder-free LIF-containing culture conditions for their capacity to give rise to undifferentiated self-renewing colonies, which generally display characteristics such as compact morphology, high AP activity, and expression of pluripotency genes. After 20 days in culture, *Ext1*$^{-/-}$ ESC clones still retained their compact dome-shaped colony morphology typical of undifferentiated ESC colonies and were morphologically indistinguishable from *Ext1*$^{+/+}$ ESCs cultured under the same conditions (Fig. 2*A*). This result suggested that HS is dispensable for the maintenance of ESC self-renewal. We further examined ESCs for AP activity and for the expression of pluripotency genes. AP assays showed that *Ext1*$^{-/-}$ ESC colonies display high AP activity equal to that of *Ext1*$^{+/+}$ ESCs (Fig. 2*B*). Quantification further showed that both *Ext1*$^{-/-}$ and *Ext1*$^{+/+}$ ESC populations contained around 95% AP-positive colonies (Fig. 2*C*). Examination of *Nanog*, *Rex-1*, and *Oct-4* transcripts showed that pluripotency genes were still expressed at high levels in *Ext1*$^{-/-}$ ESCs (Fig. 2*D*). Immunostaining for OCT-4 further showed that OCT-4 was expressed as abundantly in *Ext1*$^{-/-}$ ESCs as in *Ext1*$^{+/+}$ ESCs (Fig. 2*E*). Taken together, examinations of colony morphology, AP activity, and pluripotency gene expression consistently showed that *Ext1*$^{-/-}$ ESCs retained their normal self-renewal capacity and established that HS is not required for the maintenance of ESC self-renewal.

HS Facilitates Multilineage Cell Fate Commitment—To further explore the role of HS in self-renewal, *Ext1*$^{+/+}$ and *Ext1*$^{-/-}$ ESCs were cultured in varying concentrations of LIF. As expected, a decrease in LIF concentration correlated with fewer AP-positive colonies in *Ext1*$^{+/+}$ populations. Surprisingly, a high percentage of *Ext1*$^{-/-}$ cell colonies remained AP-positive even under low LIF concentrations, showing that *Ext1*$^{-/-}$ ESCs were unable to exit the self-renewal program (Fig. 3*A*). This was further supported by the observation that *Ext1*$^{-/-}$ cell colonies still retained their compact morphology when LIF was present at low concentrations (data not shown). To verify that HS is required for cell fate commitment into multiple lineages, ESCs were differentiated into EBs. *Ext1*$^{+/+}$ EBs developed internal cavities, a process that mirrors proamniotic cavitation of postimplantation mouse embryos. In contrast, *Ext1*$^{-/-}$ EBs failed to form cavities, showing that *Ext1*$^{-/-}$ ESCs could not

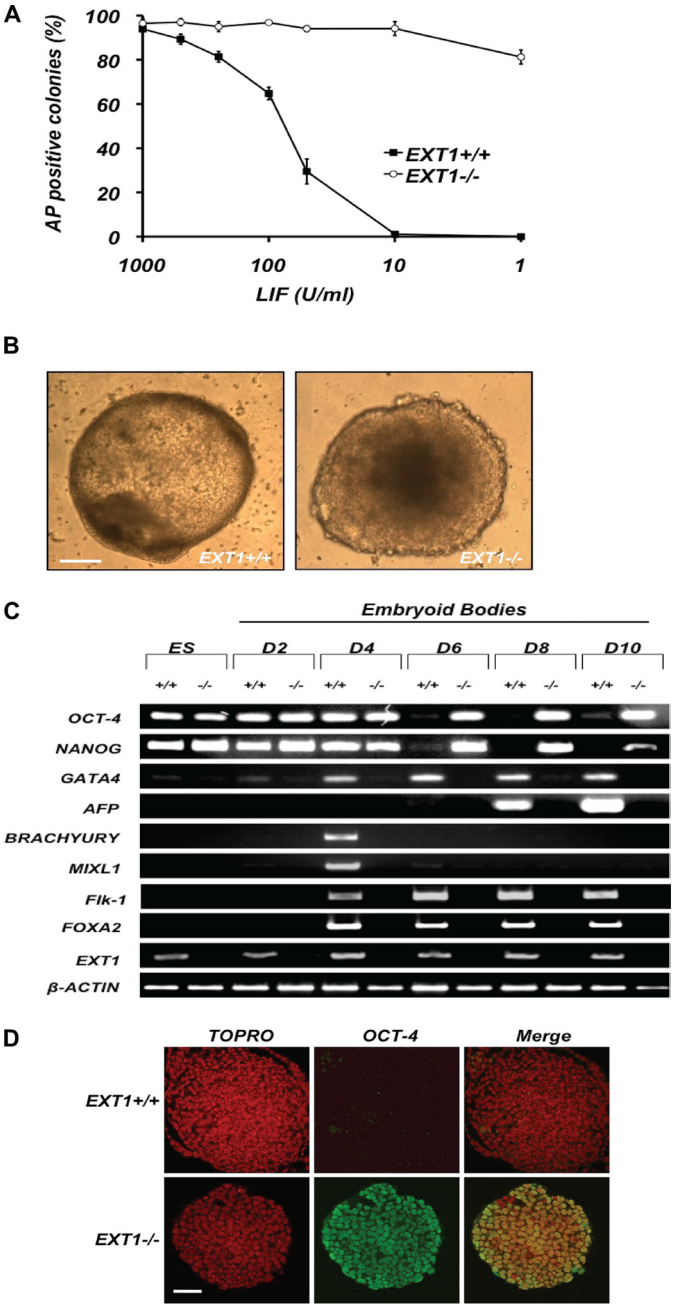


FIGURE 3. **HS is required for ESC differentiation commitment.** *A*, $Ext1^{+/+}$ and $Ext1^{-/-}$ ES cells were plated at clonal density and cultured at various LIF concentrations for 5 days. The percentage of undifferentiated colonies was examined by AP assays. *Error bars* indicate S.E. generated from triplicates of the same experiment, which is representative of at least three independent experiments. *B*, phase-contrast microscopy of day 10 EBs. *Bar*, 200 μm. *C*, RNA expression levels of pluripotency and differentiation markers during *in vitro* differentiation of ESCs. Semiquantitative RT-PCR analysis was performed on RNA extracted from either undifferentiated ESCs or EBs throughout a differentiation period of 10 days (days 2–10). β-Actin transcripts were used as an internal control. *D*, confocal microscopy images of day 8 EBs immunostained for OCT-4. *Bar*, 100 μm. *U*, units.

differentiate normally (Fig. 3*B*). During EB formation, ESCs differentiate into lineages and derivatives of all three germ layers, including endoderm, mesoderm, and ectoderm, as well as extraembryonic cell types. To determine the requirement of HS for multilineage differentiation during EB formation, transcript levels of lineage-specific marker genes and pluripotency genes were determined over the time course of EB formation. As expected, upon $Ext1^{+/+}$ EB differentiation, markers, including those of endoderm (*FoxA2*), early mesoderm (*Brachyury* and *MixL1*), late mesoderm (*Flk-1*), and extraembryonic (*Gata4* and *Afp*) lineages, became up-regulated concomitantly with a decline in transcript levels of pluripotency genes, such as *Nanog* and *Oct-4* (Fig. 3, *C* and *D*). In contrast, $Ext1^{-/-}$ EBs retained high expression levels of *Nanog* and *Oct-4*, and none of the lineage markers were expressed (Fig. 3, *C* and *D*), indicating that $Ext1^{-/-}$ ESCs failed to differentiate into multiple lineages. Taken together, these results demonstrate that HS is an essential factor for ESCs to exit from the self-renewing state and to enter multilineage cell fate commitment.

Inhibition of FGF Signaling Recapitulates the Aberrant $Ext1^{-/-}$ Cell Fate Commitment—HS-binding growth factors, including FGFs, BMPs, and Wnts, are master regulators of ESC fate decisions. FGF signaling has been shown to trigger the transition of ESCs from self-renewal to lineage commitment (7), whereas BMP-4 and Wnt3 are known to promote ESC self-renewal. Our results have shown that $Ext1^{-/-}$ ESCs retain normal self-renewal but fail to commit into developmental lineages, suggesting that HS deficiency may disrupt FGF signaling and, as a consequence, exhibit aberrant cell fate commitment. To test this idea, we examined whether inhibition of FGF signaling would recapitulate the $Ext1^{-/-}$ phenotype. ESCs were treated with the chemical FGFR inhibitor PD173074 (PD) and examined for downstream activation of ERK1/2 and changes in colony morphology upon removal of LIF. PD treatment substantially inhibited ERK1/2 phosphorylation and shows that the MAPK pathway is directly activated by FGFs in ESCs, in accordance with other reports (Fig. 4*A*) (7). Examination of colony morphologies showed that mock-treated $Ext1^{+/+}$ ESCs initiated differentiation and visibly flattened out after the onset of differentiation (Fig. 4*B*). In contrast, PD-treated $Ext1^{+/+}$ ESCs retained their compact and dome-shaped colony morphology similar to $Ext1^{-/-}$ colonies (Fig. 4*B*). Next, we assessed whether FGFR inhibition would delay the decline in OCT-4 and *Nanog* expression and suppress the expression of lineage markers. We found that OCT-4 and *Nanog* levels were substantially reduced in mock-treated $Ext1^{+/+}$ cells, whereas PD-treated $Ext1^{+/+}$ ESCs retained high levels of OCT-4 and *Nanog* that were comparable with those in $Ext1^{-/-}$ ESCs (Fig. 4, *C* and *D*). Consistent with elevated transcript levels of pluripotency genes, the expression of early differentiation markers *Wnt3*, *Brachyury*, *Nestin*, and *Gata-6* was completely suppressed by PD treatment of $Ext1^{+/+}$ ESCs. Similarly, no expression of differentiation markers was detected *in* $Ext1^{-/-}$ ESCs in the absence or presence of PD (Fig. 4, *C* and *D*). Collectively, our results show that inhibition of FGF signaling in ESCs recapitulates the $Ext1^{-/-}$ ESC lineage commitment failure, supporting our hypothesis that HS deficiency leads to defects in FGF signaling, which, in consequence, delays or blocks cell fate commitment.

HS Modulates FGF Signaling in ESCs—To directly address whether HS modulates FGF signaling in ESCs, we examined FGF-2 cell surface binding. Flow cytometry analyses detected strong cell surface binding of FGF-2 on $Ext1^{+/+}$ ESCs and no significant FGF-2 binding on $Ext1^{-/-}$ ESCs, showing that HS

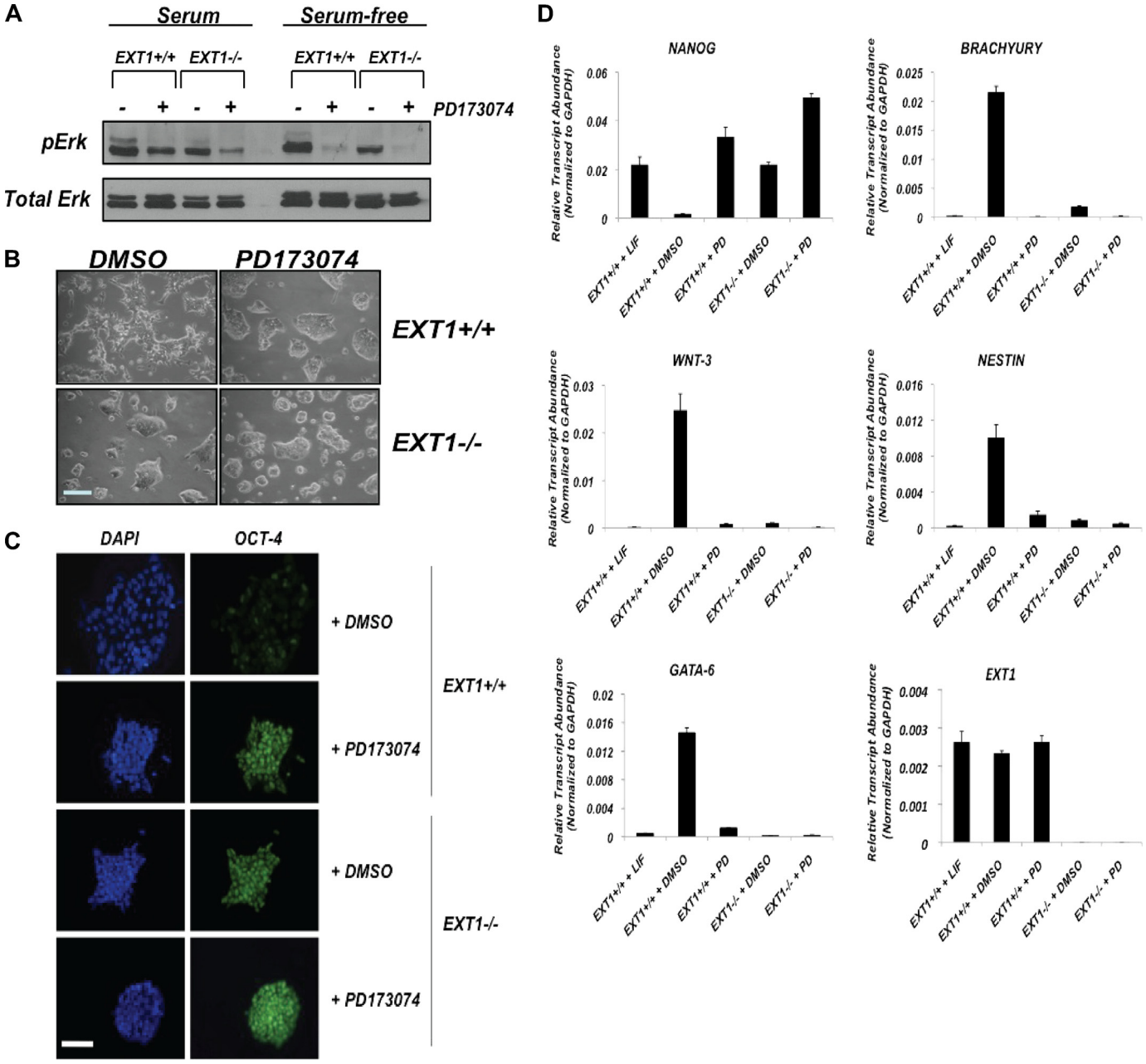


FIGURE 4. **FGFR inhibition blocks cell fate commitment and recapitulates the *Ext1*$^{-/-}$ phenotype.** *A*, ESCs were treated with 100 ng/ml PD in serum-containing or serum-free culture conditions. Western blot shows phosphorylation of ERK1/2 (*pErk*); total ERK1/2 levels were used as internal controls. *B*, ESCs were differentiated for 2 days in adherent culture in the absence of LIF with and without PD173074. Phase-contrast micrographs show colony morphology. *Bar*, 150 μm. *C*, ESCs were differentiated for 5 days in absence of LIF with PD173074 or solvent control (DMSO). Immunofluorescence staining shows OCT-4 levels. *Bar*, 150 μm. *D*, real-time RT-PCR for *Nanog*, *Wnt-3*, *Brachyury*, *Nestin*, and *Gata-6* was performed at day 5 of differentiation. Transcript levels are relative to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). *Error bars* indicate S.E. generated from triplicates of the same experiment, which is representative of at least three independent experiments. *DAPI*, 4′,6-diamidino-2-phenylindole.

deficiency disrupts efficient cell surface binding of FGF-2 (Fig. 5*A*). FGF-mediated ERK1/2 activation is the driving force behind lineage commitment (7, 31). Therefore, we tested whether activation of ERK1/2 by FGF was compromised in *Ext1*$^{-/-}$ ESCs. Stimulation of serum-starved ESCs with FGF-2 elicited substantial phosphorylation of ERK1/2 in *Ext1*$^{+/+}$ cells but not in *Ext1*$^{-/-}$ ESCs, showing directly that FGF-mediated MAPK activation is disrupted in *Ext1*$^{-/-}$ ESCs (Fig. 5*B*). In further support, we found that steady-state phospho-ERK1/2 levels were consistently lower in *Ext1*$^{-/-}$ ESCs than in *Ext1*$^{+/+}$ ESCs (Fig. 5*C*). Collectively, our data show that FGF cell surface binding and subsequent activation of the MAPK pathway are dependent on HS in ESCs.

Heparin and HS Restore Cell Fate Commitment of Ext1$^{-/-}$ *ESCs in a FGF Signaling-dependent Manner*—Heparin is a chemical analogue of HS and is commonly used as a model molecule for biological studies of HS. Heparin is well known to function as a co-factor that facilitates FGF-FGFR interactions, thereby enhancing FGF signaling in various cell types (32, 33). To support our hypothesis that HS facilitates FGF signaling to drive ESC fate commitment, we attempted to substitute endogenous HS with soluble heparin. Heparin dose-dependently

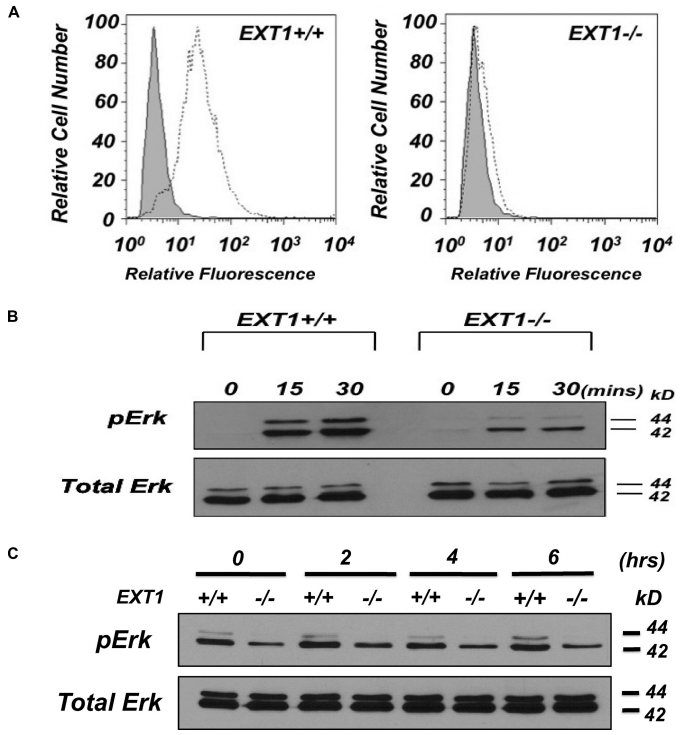


FIGURE 5. **FGF signaling is impaired in *Ext1*$^{-/-}$ ESCs.** *A*, ESCs were incubated with biotinylated FGF-2. Cell surface binding was analyzed by flow cytometry. Negative control was incubated with secondary antibody only. *B*, Western blot showing phosphorylation of ERK1/2 (*pErk*) after FGF-2 stimulation of serum-starved ESCs. Total ERK1/2 levels were used as internal controls. *C*, Western blot showing steady-state phospho-ERK1/2 levels. Lysate from ESCs cultured in presence of LIF (time point 0) and from ESCs in absence of LIF (time points 2–6 h) were analyzed. Total ERK1/2 levels were used as internal controls.

restored FGF-elicited ERK1/2 phosphorylation in *Ext1*$^{-/-}$ ESCs (Fig. 6*A*). Considering that heparin is structurally different from HS in containing a higher iduronic to glucuronic acid ratio and in being more heavily sulfated compared with HS, we further tested whether endogenous HS can, like heparin, restore FGF-dependent MAPK activity. HS isolated from *Ext1*$^{+/+}$ ESCs was supplemented to our cell culture and increased ERK1/2 phosphorylation in *Ext1*$^{-/-}$ ESCs (Fig. 6*B*). This result shows that both heparin and HS in soluble form potentiate FGF signaling of *Ext1*$^{-/-}$ ESCs and thereby restore MAPK activity to levels comparable with those of *Ext1*$^{+/+}$ ESCs.

To address whether heparin/HS could restore the competence of *Ext1*$^{-/-}$ ESCs to transit from self-renewal to differentiation, we examined OCT-4 levels upon withdrawal of LIF by immunostaining. We found that the addition of heparin as well as HS significantly reduced OCT-4 expression in *Ext1*$^{-/-}$ ESCs to levels that were similar to OCT-4 levels of *Ext1*$^{+/+}$ ESCs (Fig. 6*C*). We further examined *Ext1*$^{-/-}$ colony morphology and AP activity upon treatment with heparin/HS. As observed previously, *Ext1*$^{-/-}$ ESCs plated at clonal density retained their compact dome-shaped colony morphology after 5 days of differentiation in the absence of LIF. In contrast, heparin- and HS-treated *Ext1*$^{-/-}$ ESCs visibly flattened out and the majority of cells grew in monolayers, as did differentiated *Ext1*$^{+/+}$ cells (Fig. 6*D*). Heparin and HS treatment also resulted in loss of AP activity, suggesting that *Ext1*$^{-/-}$ ESCs no longer self-renew. Quantification showed that the number of AP-positive *Ext1*$^{-/-}$ colonies was reduced from 80% in the absence of heparin to around 5% in the presence of heparin. Similarly, HS reduced the percentage of AP-positive *Ext1*$^{-/-}$ ESCs to around 15%. Collectively, our colony morphology and AP assays demonstrated that heparin restored differentiation commitment of *Ext1*$^{-/-}$ ESCs. The heparin/HS rescue also verified that the differentiation commitment defect associated with *Ext1*$^{-/-}$ ESCs is truly due to HS deficiency alone. Furthermore, *Ext1*$^{-/-}$ ESCs were simultaneously treated with both heparin/HS and PD. In this case, PD inhibited both changes in colony morphology and reduction in AP activity, indicating that the heparin/HS-mediated rescue of cell fate commitment was achieved specifically through restoration of FGF signaling (Fig. 6*D*).

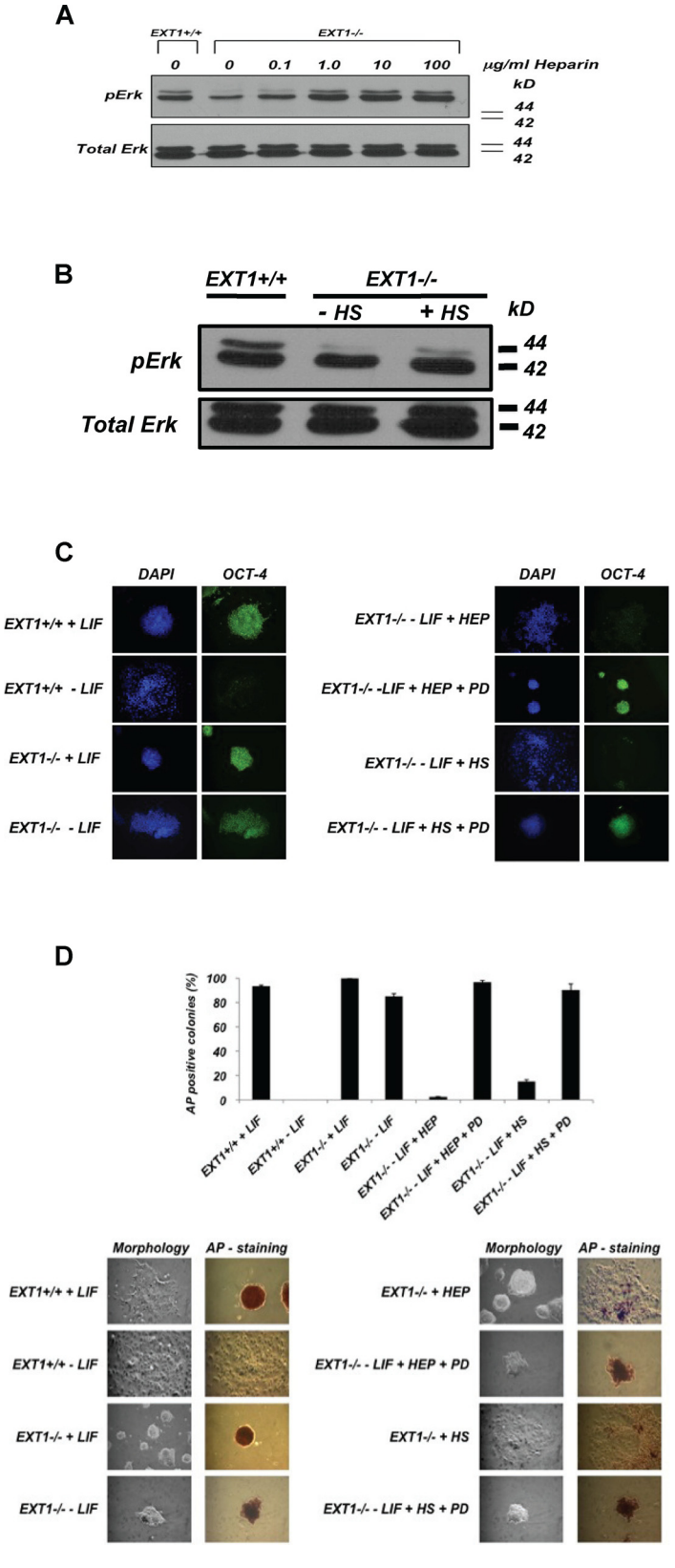
To assess whether heparin would restore lineage commitment of *Ext1*$^{-/-}$ ESCs, we analyzed transcript levels of *Nanog* and early differentiation markers, including *Wnt3*, *Brachyury*, *Nestin*, and *Gata-6*. Heparin treatment resulted in reduction of *Nanog* levels and significant increases in differentiation markers, including *Brachyury*, *Wnt3*, and *Nestin*, indicating that heparin successfully restored cell fate commitment (Fig. 6*E*). Interestingly, only *Brachyury* expression was restored to that of *Ext1*$^{+/+}$ cells. *Wnt3* and *Nestin* expression increased but not to levels of *Ext1*$^{+/+}$ ESCs, and *Gata-6* expression remained unchanged, indicating that heparin only rescued cell fate commitment and not the full differentiation potential of ESCs. Inhibition of FGF signaling by PD treatment maintained high *Nanog* levels and suppressed the expression of differentiation markers, showing again that heparin-mediated cell fate commitment rescue was achieved via restoration of FGF signaling (Fig. 6*E*).

HS Enhances FGF Signaling to Inhibit Nanog Expression, Thereby Facilitating ESC Differentiation Commitment—Our results have shown that HS modulates FGF signaling to control cell fate commitment of ESCs; however, it remains unclear how FGF signaling is integrated into the intracellular core circuitry of pluripotency-associated transcription factors. The homeoprotein Nanog is a key factor in maintaining self-renewal and acts together with Oct-4 and Sox2 to establish the ESC identity (34, 35). *Nanog* mRNA levels remained consistently high in *Ext1*$^{-/-}$ ESCs and may account for sustained self-renewal of *Ext1*$^{-/-}$ ESCs. The correlation between elevated *Nanog* expression and defects in FGF signaling of *Ext1*$^{-/-}$ ESCs lead us to hypothesize that the efficient down-regulation of *Nanog* during ESC differentiation may be directly controlled by FGF signaling. To test this hypothesis, *Ext1*$^{+/+}$ ESCs were differentiated in serum-free medium supplemented with FGF-4 in the absence of LIF for 24 h. *Nanog* mRNA levels were compared between mock- and PD-treated *Ext1*$^{+/+}$ ESCs. PD-treated ESCs maintained substantially higher *Nanog* expression compared with mock-treated cells, showing that FGF signaling directly inhibits *Nanog* expression (Fig. 6*F*). Furthermore, treatment of *Ext1*$^{-/-}$ ESCs with heparin decreased *Nanog* expression, and this effect was reversed by PD treatment (Fig. 6*F*). These results indicate a direct correlation between cell surface HS, FGF signaling, and *Nanog* expression, in which HS deficiency in *Ext1*$^{-/-}$ ESCs disrupts FGF signaling and results in elevated *Nanog* expression levels in *Ext1*$^{-/-}$ ESCs. Enhanced

Nanog expression retains self-renewal and inhibits cell fate commitment of ESCs (34, 35). Therefore, these observations indicate that HS enhances FGF signaling, which in turn inhibits *Nanog* expression, thereby facilitating the transit of ESCs from self-renewal to cell differentiation commitment.

DISCUSSION

In this study, we examined the roles of HS in self-renewal and cell fate commitment of ESCs using conditional *Ext1*$^{-/-}$ ESC lines. We found, surprisingly, that HS is not required for the maintenance of ESC self-renewal (Fig. 3, *A*–*E*). This observa-



tion is in disparity with a recent study carried out by Sasaki *et al.* (20). Employing an RNA interference-mediated gene knockdown approach, Sasaki *et al.* (20) reported spontaneous differentiation of *Ext1*-knockdown ESCs even in the presence of LIF and serum, suggesting that HS is required to maintain ESC self-renewal (20). The two opposite effects upon self-renewal observed in our study and Ref. 20 might be explained by differences in HS product. Our $Ext1^{-/-}$ ESCs were completely deficient in HS, whereas *Ext1*-knockdown cells carried residual HS with reduced chain size (20). Short HS chains may, at least in some cases, retain some of their function. For example, short HS chains were able to act as co-receptors for Wnt signaling in *Drosophila* embryos but not in embryos that lacked HS completely (14, 36, 37). Hence, it is possible that the growth factor signaling that regulates the balance between self-renewal and differentiation is modulated differently in *Ext1*-knockdown and our $Ext1^{-/-}$ ESCs, leading to the apparent discrepancies in self-renewal phenotypes.

Currently, the mechanisms that control the transition of ESCs from self-renewal to differentiation are poorly understood, but they have been suggested to involve extracellular signaling as well as epigenetic regulation (7, 8). Using suspension and adherent differentiation systems, we observed that loss of HS inhibits the transition of ESCs from self-renewal to differentiation into multiple lineages (Fig. 3), revealing HS as a critical factor that controls ESC fate commitment. This observation is supported by the studies of Johnson *et al.* (23), who reported that HS deficiency sustains *Oct-4* expression and blocks differentiation of ESCs into neural cells during directed neuronal differentiation.

Multiple HS-binding growth factors, such as Wnt, BMP, and FGF, critically regulate cell fate decisions of ESCs. Notably, Wnt and BMP signaling maintain self-renewal, whereas FGF signaling functions as an inhibitor of self-renewal and an essential factor for differentiation commitment (7, 38). In our studies, $Ext1^{-/-}$ ES cells failed to commit to lineage differentiation (Fig. 3*C*), displaying a phenotype analogous to that of ESCs deficient in FGF4 or ESCs treated with FGFR inhibitors (7, 38). Furthermore, $Ext1^{-/-}$ EBs displayed cavitation defects that have been described for $FGFR2^{-/-}$ ESCs (39). Hence, we hypothesized that defects in FGF signaling may underlie the aberrant differentiation commitment of $Ext1^{-/-}$ ESCs. Our examination of FGF cell surface binding and downstream MAPK activation demonstrated directly that HS facilitates FGF signaling in ESCs (Fig. 3). The role of FGF signaling in cell fate commitment of ESCs has been controversial. Smukler *et al.* (40) reported that FGF signaling is dispensable for commitment into a primitive neural stem cell fate. In contrast, Kunath *et al.* (7) observed that autocrine/paracrine FGF signaling was required for differentiation commitment into both neural and mesoderm lineages. To determine whether the defect in FGF signaling could recapitulate the aberrant cell fate commitment of $Ext1^{-/-}$ ESCs in our serum-containing culture conditions, we treated $Ext1^{+/+}$ ESCs with the FGFR-specific inhibitor PD173074. In agreement with previous reports (7, 41), we observed that FGF receptor inhibition results in delayed lineage commitment, phenocopying the differentiation defects of $Ext1^{-/-}$ ESCs (Fig. 4) and supporting our hypothesis that defects in FGF signaling may underlie the aberrant differentiation commitment of $Ext1^{-/-}$ ESCs. In further support, substitution with heparin or HS isolated from $Ext1^{+/+}$ ESCs restored FGF signaling in $Ext1^{-/-}$ ESCs, which correlated with effective exit from self-renewal and differentiation commitment of $Ext1^{-/-}$ ESCs (Fig. 6), and the heparin/HS-induced rescue was efficiently blocked by inhibition of FGF signaling (Fig. 6, *C–E*). Altogether, these observations consistently show that *Ext1* deficiency results in impaired FGF signaling and aberrant differentiation commitment in ESCs, establishing that HS modulates FGF signaling to control ESC differentiation commitment. Interestingly, inhibition of FGF signaling by PD treatment in $Ext1^{-/-}$ ESCs resulted in stronger reduction of ERK1/2 phosphorylation and higher *Nanog* expression when compared with PD treatment of $Ext1^{+/+}$ cells, indicating that additional ERK1/2-activating growth factors may be modulated by HS. This possibility has been supported by other studies. For example, insulin-like growth factor possesses HS-binding sites and has been shown to activate ERK1/2 in mouse ESCs (42, 43). Therefore, it will be interesting to examine whether HS modulates additional growth factors involved in facilitating cell fate commitment.

Our data established that HS facilitates FGF signaling to control ESC differentiation commitment; however, its effects on intracellular pluripotency factors are unknown. Using defined culture conditions lacking LIF, we observed that elevated *Nanog* levels in $Ext1^{-/-}$ ESCs were efficiently reduced upon heparin treatment (Fig. 6*F*), demonstrating a direct link between HS and *Nanog*. PD treatment of heparin-treated $Ext1^{-/-}$ ESCs completely reversed the reduction in *Nanog* levels, further showing that HS controls *Nanog* levels via FGF signaling. Furthermore, PD treatment of $Ext1^{+/+}$ cells resulted in the inhibition of *Nanog* down-regulation and showed directly that FGF signaling negatively controls *Nanog* expression (Fig. 6*F*). In agreement with our observation, a similar retention of *Nanog* expression upon the removal of LIF has been recently reported in ESCs treated with the MAPK inhibitor U0126 (31), suggesting that FGF signaling activates downstream MAPK to control *Nanog* levels. Enhanced *Nanog* expression has been

FIGURE 6. **Heparin/HS rescues $Ext1^{-/-}$ cell fate commitment.** *A*, Western blot showing steady-state phospho-ERK1/2 levels upon the addition of heparin in serum-containing medium. Total ERK1/2 levels were used as internal controls. *B*, as in *A* with the addition of HS isolated from $Ext1^{+/+}$ ESCs instead of heparin. *C*, ESCs were differentiated for 5 days. $Ext1^{-/-}$ ESCs were treated with (+) or without (−) heparin (*HEP*), HS, and PD. Shown are fluorescence microscopy pictures of OCT-4 staining. *Bar*, 200 μm. *D*, ESCs were plated at clonal density and cultured for 5 days in the absence of LIF supplemented with heparin or HS and/or PD. Subsequently, colonies were analyzed for AP activity. The proportion of AP-positive colonies was quantified. *Error bars* indicate the S.E. generated from triplicates of the same experiment, which is representative of at least three independent experiments. In parallel, colony morphology was examined by phase-contrast microscopy. *Bar*, 200 μm. *E*, real-time RT-PCR for *Nanog*, *Wnt-3*, *Brachyury*, *Nestin*, and *Gata-6* was performed at day 5 of differentiation. Transcript levels are relative to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). *Error bars* indicate S.E. generated from triplicates of the same experiment, which is representative of at least three independent experiments. *F*, ESCs were transferred into serum-free medium supplemented with 10 ng/ml FGF-4 with or without PD. $Ext1^{-/-}$ ESCs were treated with 1 μg/ml heparin in the absence or presence of PD. After 24 h, RNA was collected, and *Nanog* mRNA levels were examined by real-time RT-PCR.

shown to maintain self-renewal and to inhibit differentiation of ESCs (34, 35). Therefore, our study delineates that HS promotes ESC exit from self-renewal and cell fate commitment by inhibiting *Nanog* expression via facilitation of FGF signaling.

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