Relative luciferase activity is defined as the ratio of luciferase activity of TOPFLASH to that of FOPFLASH.

For immunostaining of β-catenin, mES cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% saponin 3 days after transfection. After washing and subsequent blocking, cells were stained with an anti-β-catenin antibody. After washing, cells were stained with fluorescein isothiocyanate-conjugated secondary antibody (Chemicon) and counterstained with propidium iodide (BD Biosciences). Immunofluorescence images were taken using an LSM5Pascal confocal laser scanning microscope (Carl Zeiss) with ×40/1.3 objectives at room temperature.

^{125}I-Labeled Wnt3a Binding Assay—Two micrograms of recombinant mouse Wnt3a (R&D Systems) were iodinated with 100 μCi of ^{125}I-sodium (ARC) using iodogen-precoated reaction tubes (Pierce) according to manufacturer's instructions. Radiolabeled Wnt3a was separated from unincorporated ^{125}I-sodium on a PD-10 column. The specific activity of the radiolabeled Wnt3a was 1.85×10^4 cpm/ng protein. For the binding assay, mES cells were harvested 2 days after transfection and replated in triplicate at 5×10^5 cells per well in 24-well 0.2% gelatin-coated plates. The cells were allowed to attach for 3 h and then washed three times with ice-cold binding buffer (serum-free ES medium containing 1 mg/ml BSA and 0.1% sodium azide). After incubating with 80 ng/ml ^{125}I-labeled Wnt3a either alone or in the presence of 100 μg/ml heparin (Sigma) in binding buffer for 3 h at 4 °C, the cells were gently washed three times with ice-cold binding buffer and lysed with 0.2 N NaOH. The amount of radiolabeling in each extract was counted with a gamma counter (Aloka).

Surface Plasmon Resonance (SPR) Analysis—Heparin (Nacalai Tesque) was dialyzed against distilled water using an MWCO3500 membrane (SpectroPore) and lyophilized. Then the heparin was conjugated with a monovalent linker molecule to prepare the ligand conjugate for the immobilization of heparin on the gold-coated chip as previously described (31). The surface of the gold-coated chip (SUDx-Biotec) was oxidatively washed with UV ozone cleaner (Structure Probe Inc.) for 20 min. The chips were then immersed in 1 μM of the ligand-conjugate in 50% (v/v) methanol solution overnight at room temperature with gentle agitation to prepare Sugar Chips with immobilized heparin. The Sugar Chips were washed sequentially with water, PBS containing 0.05% Tween 20 and water, and dried at room temperature.

The Sugar Chip with immobilized heparin was set on a prism with refraction oil (n_D = 1.518, Cargill Laboratories Inc.) in an SPR apparatus (SPR670M, Moritex). The SPR measurements were performed at room temperature in accordance with the manufacturer's instructions and using Tris-buffered saline (20 mM Tris-HCl, 150 mM NaCl, pH 7.4) containing 0.05% Tween 20 and 0.1% BSA as the running buffer at a flow rate of 15 μl/min. The kinetic binding parameters were calculated using the software of the manufacturer. We performed binding of BSA or von Willebrand factor (vWF) A1 to heparin, as negative and positive controls, respectively (supplemental data 1) (apparent association and dissociation rate constants k_a (M^{-1} s^{-1}) and k_d (s^{-1}) of vWF A1, 3.51×10^3, 4.38×10^{-3}, respectively; apparent equilibrium constant K_D (nM) for binding of vWF A1 to heparin, 1248.1).

RT-PCR and Real Time PCR—Total RNA was isolated from mES cells by TRIzol Reagent (Invitrogen) and subsequently reverse transcribed using an oligo-dT primer (Invitrogen) and a SuperscriptII first strand synthesis kit (Invitrogen). Primer sets for PCR amplification are listed in supplemental data 2. All cDNAs were amplified in quantitative ranges, which were confirmed by examining various cycles for the samples giving maximum levels of signals in each primer sets. Primers sets and probes for real time PCR are listed in supplemental data 3. Real time PCR was performed using an ABI PRISM® 7700 sequence detection system (Applied Biosystems). The relative amounts of each mRNA were normalized by *β-actin* mRNA in the same cDNA.

RESULTS

HS Chains Are Reduced by Knockdown of EXT1 mRNA—Hereditary multiple exostosis is an autosomal dominant disorder characterized by the development of benign cartilage-capped tumors at the juxta-epiphyseal regions of long bones (32). It is associated with mutation of either *EXT1* or *EXT2*, which encode glycosyltransferases possessing both D-glucuronic acid and GlcNAc-transferase activities that are necessary for HS chain elongation (33, 34). In the Golgi apparatus, EXT1 and EXT2 form a hetero-oligomeric complex that exhibits higher glycosyltransferase activity than either enzyme alone (35, 36). To examine the effect of reduced HS chain levels in ES cells, we knocked down expression of *EXT1* mRNA using RNAi. We designed two constructs expressing different siRNAs targeting *EXT1* (*EXT1-1* and *EXT1-2*), as described previously (29), and siRNAs targeting *EGFP* as a negative control. We represent mES cells transfected with *EGFP* siRNA expression plasmids as "control cells" throughout this paper. Real time PCR analysis performed 2 days after transfection of the cells with *EXT1* siRNA expression plasmids showed that the level of *EXT1* mRNA was reduced to about 20% that of control cells. In contrast, the expression of other nontargeted genes was unaffected by this treatment in the two mES lines tested (R1 and E14TG2a) (Fig. 1*A*). The knockdown effect on *EXT1* was maintained for at least 3 days following puromycin selection. We used E14TG2a to confirm the same knockdown effect on mES cells using one siRNA expression plasmid (*EXT1-1*) throughout all experiments.

Two days after transfection, we performed FACS analysis using an anti-HS antibody to determine whether HS chain expression was reduced by knockdown of *EXT1* mRNA. As shown in Fig. 1*B*, HS chains were abundant in mES cells, whereas CS chains were not. We observed a significant reduction in HS chain expression on the cell surface of cells transfected with an *EXT1* siRNA expression plasmid compared with control cells (Fig. 1*B*). The knockdown effect was higher using *EXT1-1* siRNA compared with *EXT1-2* siRNA. Reduction of HS chain expression was accompanied by a subtle increase in CS chain synthesis that correlated with the level of reduction of HS chains. Such an increase in CS chain expression was previously observed in *EXT1*-deficient ES cells derived from *EXT1* knock-out mice (25). The reduction of HS chain expression in

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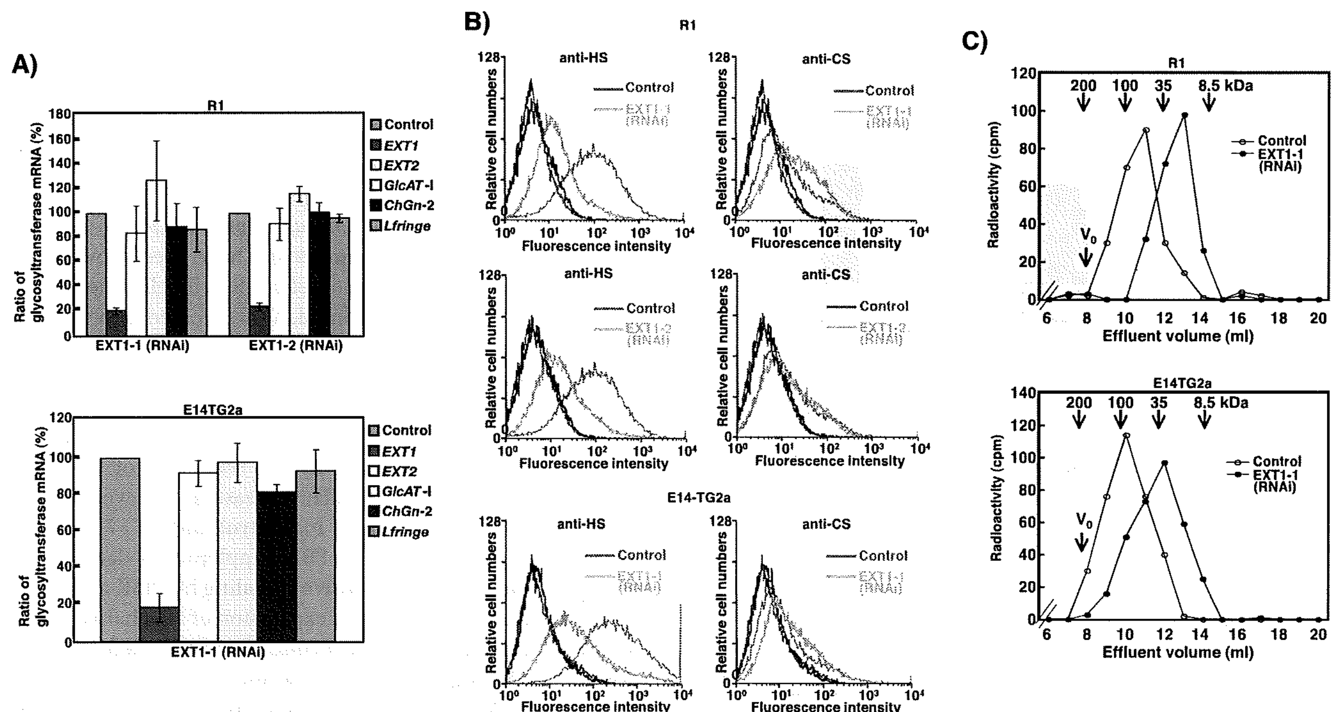

FIGURE 1. ***EXT1* siRNA induces efficient knockdown of *EXT1* mRNA and reduction of HS chains in mES cells.** *A*, real time PCR analysis of cells 2 days after transfection. The results are shown as the proportion (%) of expression of each glycosyltransferase mRNA relative to that observed in control cells: *GlcAT*, glucuronosyltransferase; *ChGn*, chondroitin β1,4-*N*-acetylgalactosaminyltransferase; *Lfringe*, lunatic fringe. The values shown are the means ± S.D. of three independent experiments. We used two constructs expressing different siRNAs targeting *EXT1* (*EXT1-1* and *EXT1-2*). *B*, FACS analysis of cells 2 days after transfection using an anti-HS antibody or anti-CS antibody (*black* and *blue lines* represent IgM isotype control of control and *EXT1*-deficient cells, respectively). Three independent experiments were performed and representative results are shown. *C*, molecular size analysis of HS chains from mES cell surface by gel chromatography on a Sephacryl S-300 column. The *open squares* and *closed squares* indicate the elution profiles of HS chains from control and *EXT1*-deficient cells, respectively. *Arrows* indicate the elution positions of dextran molecular mass standards.

EXT1-deficient cells was also confirmed by HPLC analysis of unsaturated disaccharides from HS chains (data not shown). We also examined the molecular sizes of HS chains derived from *EXT1*-deficient cell surface by gel chromatography. As is shown in Fig. 1*C*, the lengths of HS chains in *EXT1*-deficient cells (~35kDa) appeared to be reduced compared with control cells (50–150 kDa).

HS Chains Are Important for Self-renewal and Proliferation of mES Cells—We performed colony assays on *EXT1*-deficient cells to determine whether the resulting reduction in HS chain expression affected the frequency with which the cells remain in an undifferentiated state. The number of colonies derived from *EXT1*-deficient cells that remained in an undifferentiated state fell to about 50% that of control cells, even in the presence of LIF and serum (Fig. 2*A*). We next examined proliferation of transfected mES cells. As shown in Fig. 2*B*, the rate of proliferation of *EXT1*-deficient cells decreased significantly compared with control cells. These results suggest that the reduction in HS chain expression inhibited the capacity for both self-renewal and proliferation of mES cells, presumably because of reduced levels of HS chain-dependent signaling.

The Reduction of HS Chain Expression Increases Spontaneous Differentiation of mES Cells into Extraembryonic Endoderm—We compared the morphologies of control and *EXT1*-deficient cells. Four days after transfection in the presence of LIF, control cells appeared to be undifferentiated cells with modest packed morphology in which the cells preferentially adhered to one another, but differentiated cells with a flattened morphology also existed at low levels because of the feeder-free culture conditions (Fig. 3*A*). In contrast, almost all of the *EXT1*-deficient cells exhibited a flattened, differentiated morphology that, in some cells, was reminiscent of the stellate morphology of the parietal endoderm (37) (Fig. 3*A*). Furthermore, expression of *Oct3/4* and *Nanog*, which are markers of the undifferentiated state, was significantly decreased in *EXT1*-deficient cells compared with control cells (Fig. 3*B*), indicating that spontaneous differentiation of *EXT1*-deficient cells occurred more frequently than of control cells. Upon withdrawal of LIF, control cells exhibited a variety of flattened and differentiated morphologies (data not shown), suggesting that pluripotency had been maintained. However, most of the *EXT1*-deficient cells exhibited a parietal endoderm-like morphology (data not shown), as was the case in the presence of LIF.

To further characterize the transfected mES cells, we evaluated expression of several germ layer markers by real time PCR analysis of cells 4 days after transfection (Fig. 3, *B–D*). In the presence of LIF, we detected higher expression in *EXT1*-deficient cells of markers of the extraembryonic endoderm lineage (primitive endoderm, *Gata4* and *Gata6*; parietal endoderm, *Dab2* and *LamininB1*; and visceral endoderm, *Bmp2* and *Ihh*) than control cells (Fig. 3*C*), whereas other lineage markers (such as the trophoblast marker, *Cdx2*; the primitive ectoderm marker, *Fgf-5*; the neuroectoderm marker, *Isl1*; and the mesoderm marker, *Brachyury*) were weakly expressed (Fig. 3*D*).

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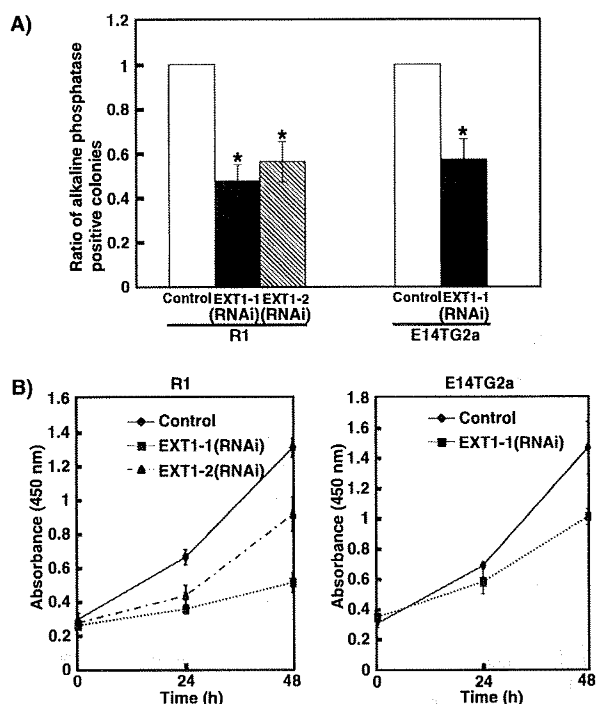

FIGURE 2. ***EXT1*-deficient cells show decreased potential for self-renewal and proliferation.** *A*, self-renewal assay. The ratio of AP-positive colonies is shown after normalization against control cells (value = 1). Approximately 70% of the colonies derived from the control cells remained in an undifferentiated state in feeder-free culture. The values shown are the means ± S.D. from three independent experiments, and significant values are indicated; *, $p < 0.01$, in comparison with control. *B*, proliferation assay. The values shown are the means ± S.D. from three independent experiments.

These results reflect the morphologies of *EXT1*-deficient cells as shown in Fig. 3*A*. Following withdrawal of LIF, *EXT1*-deficient cells underwent further differentiation. We observed only induction of extraembryonic endoderm lineage markers in these cultures compared with control cells (Fig. 3, *C* and *D*). Upon induction of differentiation of control cells, various markers of differentiation, including *Cdx2*, *Fgf-5*, and *Brachyury*, and extraembryonic endoderm lineage markers exhibited a further increase in expression associated with a decrease of expression of *Oct3/4* and *Nanog*, indicating that pluripotency had been maintained. Our analyses indicate that HS chains are important for the maintenance of the undifferentiated state and of pluripotency of mES cells and that signaling pathways mediated by HS chains may be involved in signaling pathways that control the differentiation of mES cells into the extraembryonic endoderm lineage.

We next examined the formation of EBs in suspension culture in control and *EXT1*-deficient cells. EB formation occurs during the *in vitro* differentiation of ES cells into the three germ layers, mesoderm, ectoderm, and endoderm. This differentiation process resembles the formation of the developing conceptus at the egg-cylinder stage. As shown in Fig. 3, *E* and *F*, almost all control cells formed large fluid-filled cystic EBs 6 days after EB formation, in which differentiation into the three germ layers had begun to proceed. In contrast, 53.02 ± 5.83% of *EXT1*-deficient cells formed noncystic and significantly small EBs. This suggests that the reduction in HS chain expression inhibited differentiation of *EXT1*-deficient cells into the three germ layers, supporting the results that differentiation of *EXT1*-deficient cells into nonextraembryonic endoderm lineage was inhibited in normal culture (Fig. 3, *D* and *E*). Similar results were obtained in cells transfected with the second siRNA expression plasmid targeting *EXT1* (*EXT1-2*) and also in a second mES cell line (E14TG2a; data not shown).

The Reduction of HS Chains Down-regulates Specific Signaling—Several signaling molecules have been shown to be important for the maintenance of mES cell self-renewal, for example, LIF/STAT3, BMP/Smad, Wnt/β-catenin, phosphoinositide 3-kinase (PI3K)/Akt, and members of the Src family (7–12, 38, 39). Therefore, we performed Western blot analysis of cell lysates prepared 2 days after transfection of control and *EXT1*-deficient cells to determine whether the activity of signaling molecules was affected by the reduction of HS chain expression. We observed a similar increase in the level of phosphorylation of Akt and Smad1 in control and *EXT1*-deficient cells following exposure to 15% FBS (supplemental data 4), suggesting that the serum-responsive signaling by PI3K and Smad was not affected by the reduced expression of HS chains. Moreover, we found that treatment of control or *EXT1*-deficient cells with LIF or BMP4 gave rise to similar increases in the phosphorylation of STAT3 and Smad1, respectively (Fig. 4, *A* and *B*). Heparitinase treatment was performed to examine the effect of HS chain depletion on BMP/Smad and LIF/STAT3 signaling. This treatment led to a reduction in BMP/Smad signaling but not of LIF/STAT3 signaling (Fig. 4, *C* and *D*), demonstrating that HS chains contribute to BMP signaling but not LIF signaling in mES cells. The level of phosphorylation of ERK in response to basic FGF treatment was reduced in *EXT1*-deficient cells compared with the level observed in control cells, suggesting that FGF signaling was reduced in *EXT1*-deficient cells (Fig. 4*B*) and more reduction was detected after heparitinase treatment (Fig. 4, *C* and *D*). Phosphorylation of the Src family member, cYes, which has been implicated in the maintenance of self-renewal of ES cells (38), was activated to a similar extent in both control and *EXT1*-deficient cells following treatment with LIF and FBS (Fig. 4, *A* and *B*). We observed comparable results in similar studies with E14TG2a cells (data not shown).

HS Chains Regulate Autocrine/Paracrine Wnt/β-Catenin Signaling in mES Cells—In the absence of feeder cells, we observed a significantly higher level of phosphorylation of β-catenin in *EXT1*-deficient cells than control cells (Fig. 5*A*). This suggests that there was a decrease in autocrine/paracrine Wnt/β-catenin signaling in *EXT1*-deficient cells. Next, we examined Wnt/β-catenin signaling using a luciferase reporter system. We found a significant decrease in luciferase activity in *EXT1*-deficient cells compared with control cells under feeder-free culture conditions (Fig. 5*B*). This is consistent with a reduction in autocrine/paracrine Wnt/β-catenin signaling in *EXT1*-deficient cells. We subsequently examined the nuclear localization of β-catenin, an indicator of activation of the canonical Wnt pathway. Accumulation of β-catenin was significantly decreased in the nuclei of *EXT1*-deficient cells compared with control cells (supplemental data 5). We carried out a Western blot analysis and confirmed that nuclear accumula-

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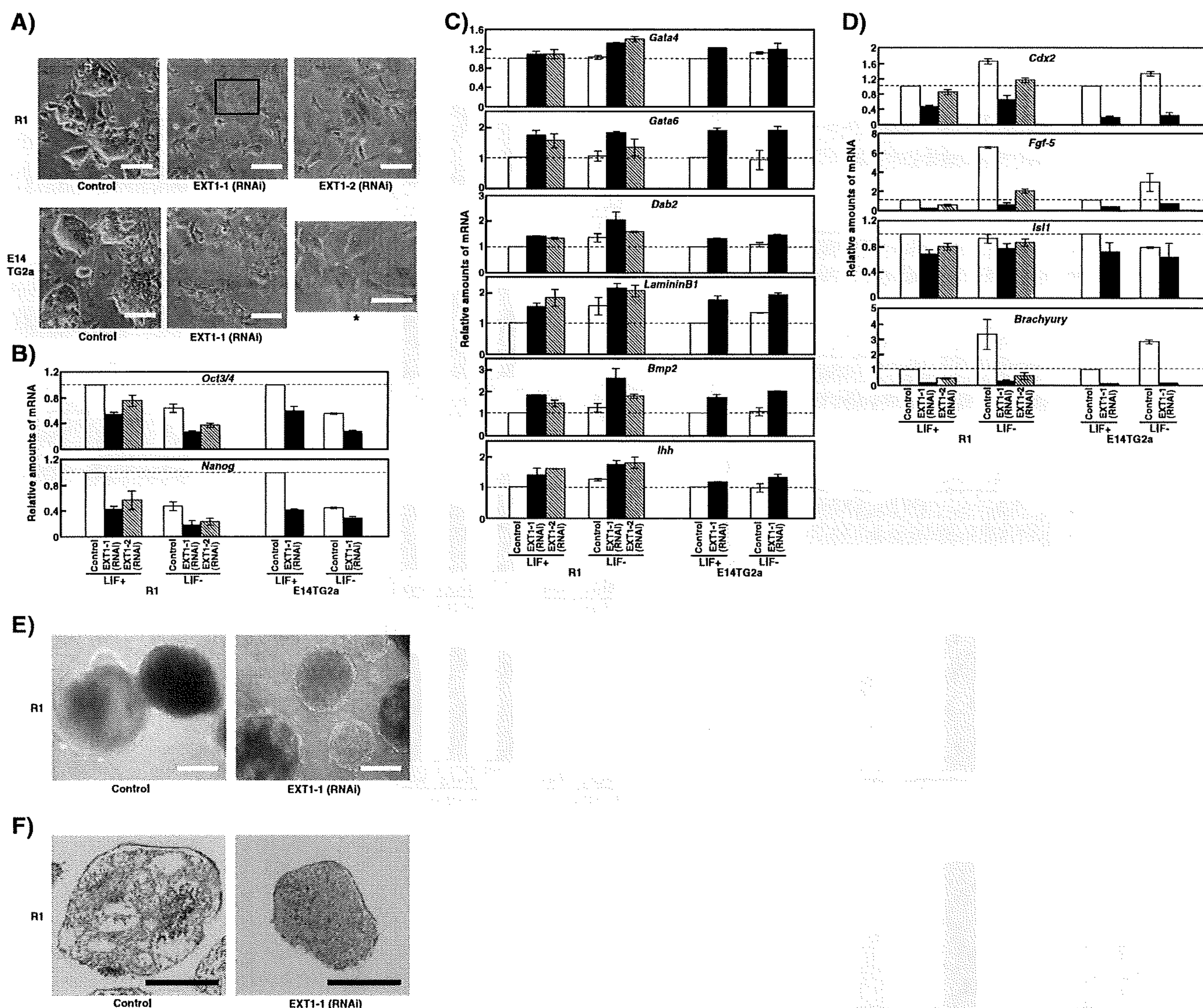

FIGURE 3. ***EXT1*-deficient cells differentiate spontaneously into the extraembryonic endoderm lineage.** *A*, photomicrographs of cells at 4 days after transfection in the presence of LIF. Representative photographs of control and *EXT1*-deficient cells from two independent experiments are shown. *Scale bars*, 100 μm. (*, high magnification image of the *boxed area* in *EXT1*-deficient R1 cells, stellate morphology of parietal endoderm. *Scale bars*, 50 μm). *B–D*, real time PCR analysis of cells 4 days after transfection in the presence or absence of LIF. The results are shown after normalization against control cells in the presence of LIF (value = 1). The values shown are the means ± S.D. from two independent experiments. *E*, representative photomicrographs at 6 days after EB formation. *Scale bars*, 200 μm. *F*, representative hematoxylin/eosin sections of EBs at 6 days after EB formation. *Scale bars*, 200 μm.

tion of β-catenin was reduced in *EXT1*-deficient cells compared with control cells (Fig. 5*C*). Thus, activation of Wnt/β-catenin signaling appeared to be reduced in *EXT1*-deficient cells. Similar results were observed in analogous studies using E14TG2a cells (data not shown).

RT-PCR analysis showed that both R1 and E14TG2a cell lines expressed several *Wnts*, as did MEFs. *Wnt2*, *Wnt4*, and *Wnt5a* were expressed in all cells, but *Wnt3a*, *Wnt7a*, and *Wnt10b* were expressed only in the mES cells (Fig. 5*D*). We examined the effect of reduction of HS chains on the ability of Wnt3a to bind to the mES cell surface. *EXT1*-deficient cells exhibited a significantly lower specific ^{125}I-Wnt3a binding than control cells (Fig. 5*E*), indicating that HS chains contribute to the binding of Wnt3a to the mES cell surface. Furthermore, SPR analysis (supplemental data 6) confirmed binding of Wnt3a to heparin, the structural analogue of HS chains, indicating that Wnt3a does indeed bind to HS chains on mES cells (apparent association and dissociation rate constants k_a (M^{-1} s^{-1}) and k_d (s^{-1}), 2.22 × 10^5, 5.77 × 10^{-3}, respectively, apparent equilibrium constant K_D (nM) for binding of Wnt3a to heparin, 26.0). These results demonstrate that HS chains regulate autocrine/paracrine Wnt/β-catenin signaling in mES cells.

HS Chain Regulation of Wnt/β-Catenin Signaling Contributes to Self-renewal of mES Cells—To determine whether the regulation of Wnt/β-catenin signaling by HS chains is necessary for the self-renewal and pluripotency of mES cells, we examined the effect of exogenous activation of Wnt/β-catenin signaling in *EXT1*-deficient cells using BIO, a specific pharmacological inhibitor of glycogen synthase kinase-3. The level of luciferase activity of Wnt/β-catenin signaling in BIO-treated

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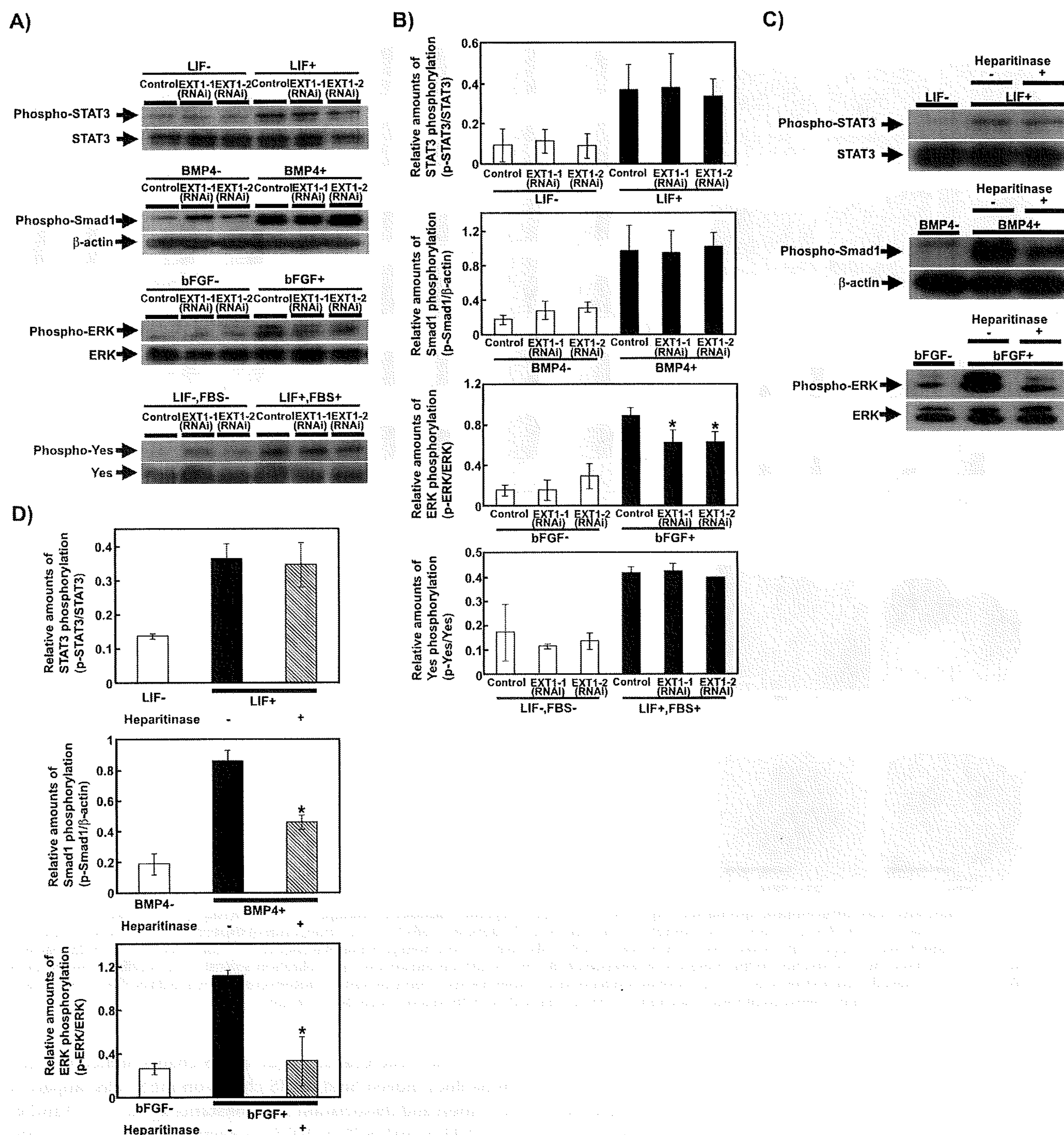

FIGURE 4. **Specific signaling is decreased in *EXT1*-deficient cells.** *A*, Western blot analysis of mES cells stimulated with each extrinsic factor. Two independent experiments were performed, and representative results are shown. *B*, quantification of Western blots shown in *A*. The *histograms* show mean densitometric readings ± S.D. of the phosphorylated protein/loading controls. Values were obtained from duplicate measurements of two independent experiments and significant values are indicated; *, $p < 0.01$, in comparison with stimulated control. *C*, Western blot analysis of mES cells stimulated with each extrinsic factor after treatment with or without heparitinase. Two independent experiments were performed, and representative results are shown. *D*, quantification of Western blots shown in *C*. The *histograms* show mean densitometric readings ± S.D. of the phosphoprotein/loading controls. Values were obtained from duplicate measurements of two independent experiments, and significant values are indicated; *, $p < 0.01$, in comparison with heparitinase untreated and extrinsically stimulated samples.

cells was 2–3-fold higher than in cells treated with MeBIO, an inactive analogue of BIO (data not shown). Two days after transfection, the level of *Nanog* mRNA was significantly up-regulated in BIO-treated cells (Fig. 6*A*), indicating that *Nanog* expression is regulated by Wnt/β-catenin signaling. The levels of *Nanog* and *Oct3/4* mRNAs in MeBIO-treated *EXT1*-deficient cells were 30–50% of those in MeBIO-treated control cells (Fig. 6*A*). Untreated cells showed similar results (data not

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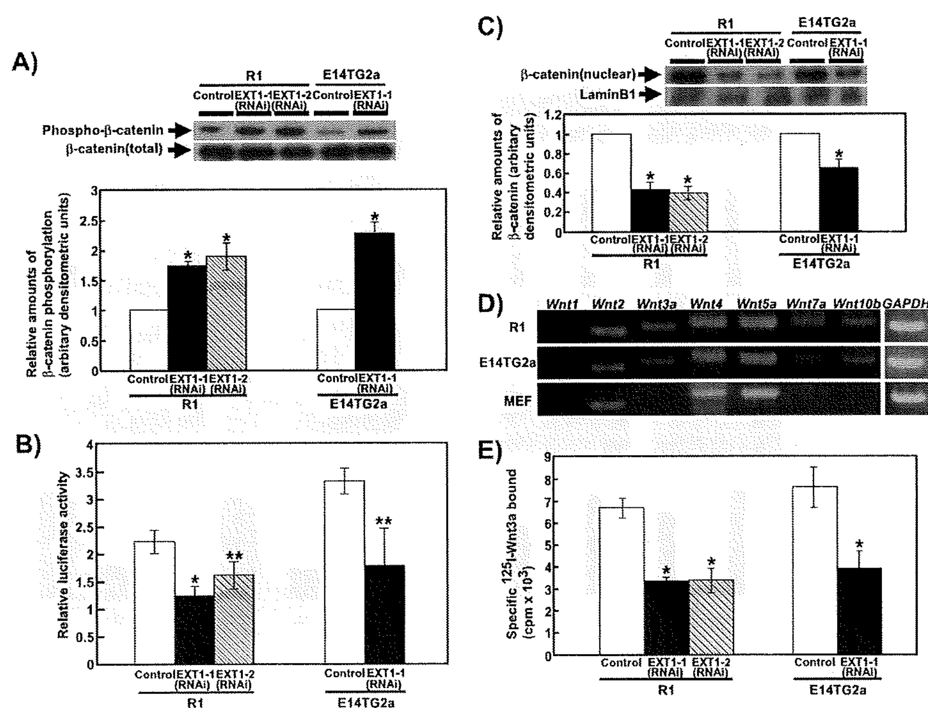

FIGURE 5. **Wnt/β-catenin signaling is decreased in *EXT1*-deficient cells.** *A*, Western blot analysis of mES cells after LIF and serum starvation for 4 h at 2 days after transfection. Representative immunoblots are shown. The *histograms* show mean densitometric readings ± S.D. of the phospho-β-catenin/total β-catenin after normalization against control cells (value = 1). Values were obtained from duplicate measurements of two independent experiments, and significant values are indicated; *, $p < 0.01$, in comparison with control. *B*, luciferase reporter assay. Relative luciferase activities (TOPFLASH/FOPFLASH) are shown as means ± S.D. from three independent experiments, and significant values are indicated; *, $p < 0.01$; **, $p < 0.05$, in comparison with control. *C*, Western blot analysis of mES cells at 3 days after transfection. Representative immunoblots are shown. The *histograms* show mean densitometric readings ± S.D. of the β-catenin/lamin B_1 after normalization against control cells (value = 1). Values were obtained from duplicate measurements of two independent experiments and significant values are indicated; *, $p < 0.01$, in comparison with control. *D*, RT-PCR analysis of the expression of several *Wnts* in mES cells and MEFs. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase. *E*, binding assay of ^{125}I-labeled Wnt3a. The value of specific ^{125}I-Wnt3a binding (total counts/min bound minus counts/min bound in the presence of 100 μg/ml free heparin) is the mean ± S.D. of three independent experiments, and significant values are indicated; *, $p < 0.01$, in comparison with control.

shown). This suggests that decreased signaling in *EXT1*-deficient cells affects the expression of *Nanog* and *Oct3/4*. BIO treatment rescued the level of *Nanog* mRNA in *EXT1*-deficient cells, but not of *Oct3/4* (Fig. 6*A*). The signaling pathways that regulate *Oct3/4* expression are therefore dependent upon HS chain expression but not upon Wnt. Furthermore, the expression patterns of Nanog and Oct3/4 proteins were correlated with mRNA levels (Fig. 6*B*). Recently, it has been demonstrated that the orphan nuclear receptor LRH-1 is required for maintenance of *Oct3/4* expression in mES cells (40). The level of *LRH-1* mRNA in *EXT1*-deficient cells treated with MeBIO was also reduced to 30–50% that in control cells (Fig. 6*A*), similarly to *Oct3/4* expression, but was unaffected by BIO treatment (Fig. 6*A*). This suggests that the reduction in *Oct3/4* expression in *EXT1*-deficient cells is mediated by LRH-1. Overall, our analyses demonstrate that Wnt/β-catenin signaling through HS chains regulates Nanog expression but not that of Oct3/4 in mES cells.

Next, we examined the ability of BIO to rescue self-renewal in *EXT1*-deficient cells. The number of AP-positive colonies derived from *EXT1*-deficient cells treated with MeBIO was significantly reduced compared with that of control cells (Fig. 6*C*). Nearly all of the colonies derived from culture of *EXT1*-deficient cells in the presence of BIO exhibited a tightly packed and AP-positive morphology reminiscent of undifferentiated ES cells cultured on a feeder layer. BIO treatment therefore rescued defective self-renewal in *EXT1*-deficient cells (Fig. 6*C*). This rescue effect demonstrates that HS chain-dependent signaling by Wnt/β-catenin contributes to self-renewal of mES cells. However, the total numbers of colonies scarcely differed between BIO-treated cells and MeBIO-treated cells (Fig. 6*C*), indicating that BIO treatment had no effect on cell proliferation. In a proliferation assay, we observed no effect of BIO on mES cell proliferation compared with untreated and MeBIO-treated cells (data not shown). These results demonstrate that HS chain-dependent signaling by autocrine/paracrine Wnt/β-catenin is required for the maintenance of self-renewal but not for the proliferation of mES cells.

Wnt/β-Catenin Signaling Regulated by HS Chains Is Important for Pluripotency of mES Cells—Our next step was to examine the effect of BIO on *EXT1*-deficient cell pluripotency in the absence of LIF, in which ES cells spontaneously differentiated into several lineages (Fig. 3, *B–D*). The expression of differentiation markers was analyzed 4 days after transfection using real time PCR analysis (Fig. 6, *D–F*). The expression of various differentiation markers was examined in *EXT1*-deficient cells treated with either BIO or MeBIO. BIO induced expression of the markers, including *Cdx2*, *Fgf-5*, and *Brachyury*; expression of the differentiation markers was at a low level in the MeBIO-treated cells. In the latter treatment group, only markers of the extraembryonic endoderm lineage (*Gata6*, *LamininB1*, and *BMP2*) were detected at appreciable levels (similar to those described in Fig. 3, *C* and *D*). Therefore, BIO rescued the defective pluripotency of *EXT1*-deficient cells.

Nanog-deficient mES cells spontaneously differentiate into the extraembryonic endoderm lineage, implicating Nanog in the control of mES cell pluripotency (14, 15). *Nanog* and *Oct3/4* expression was reduced in *EXT1*-deficient cells treated with MeBIO, whereas treatment with BIO rescued the level of *Nanog* expression although only to the same level as in MeBIO-treated control cells (Fig. 6*D*). Thus Wnt/β-catenin signaling sustains *Nanog* expression. In control cells, BIO treatment resulted in reduced expression of various differentiation markers associated with maintenance of *Nanog* and *Oct3/4* expression com-

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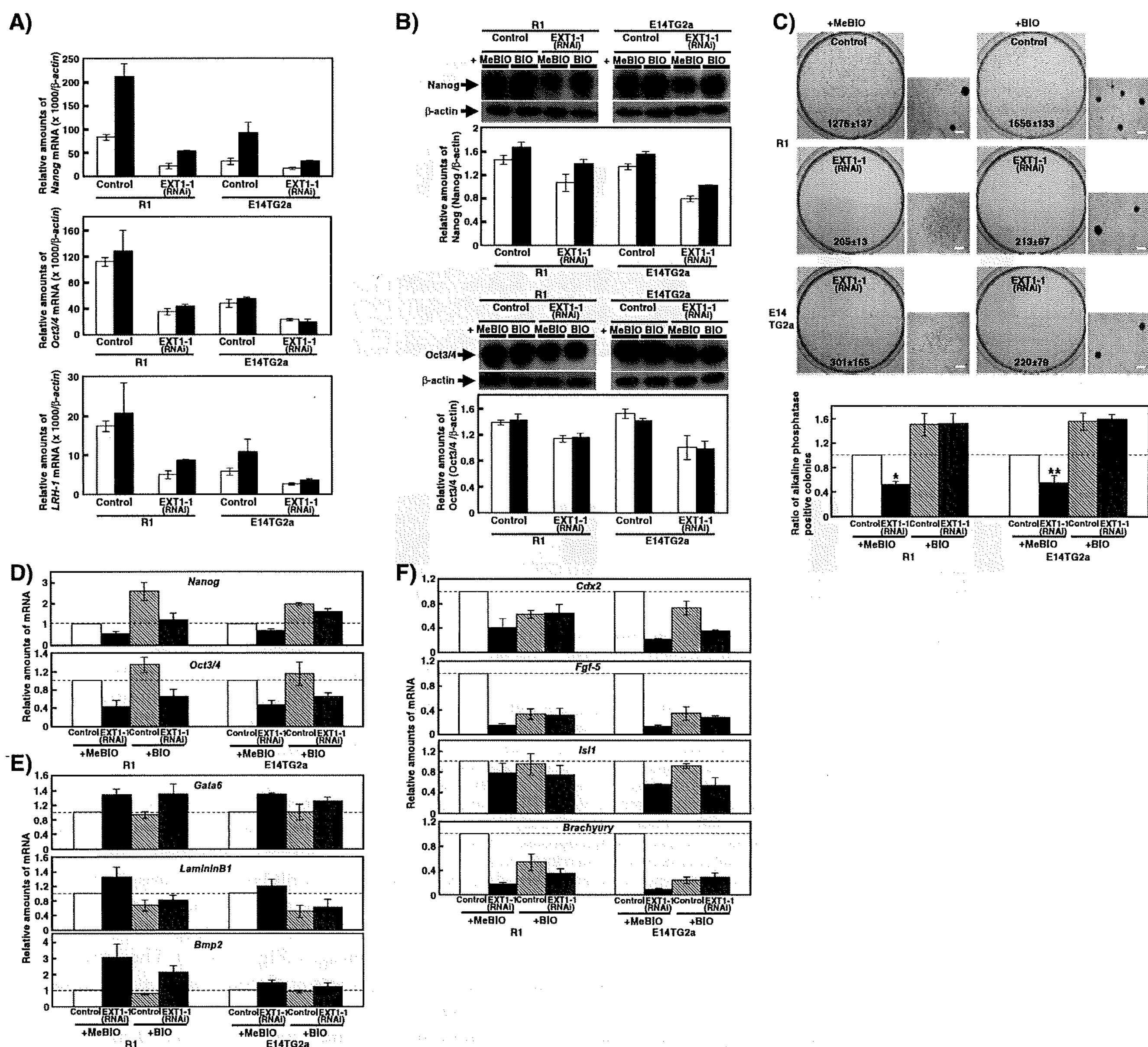

FIGURE 6. **Activation of Wnt/β-catenin signaling by treatment with BIO rescues self-renewal and pluripotency of *EXT1*-deficient cells.** *A*, real time PCR analysis of mES cells 2 days after transfection. The value shown is the mean ± S.D. of three independent experiments (*white bar*, MeBIO-treated; *black bar*, BIO-treated). *B*, Western blot analysis of mES cells at 2 days after transfection. Representative immunoblots are shown. The *histograms* show mean densitometric readings ± S.D. of the protein/β-actin. Values were obtained from duplicate measurements of two independent experiments (*white bar*, MeBIO-treated; *black bar*, BIO-treated). *C*, self-renewal assay. *Upper panels*, photographs of representative colonies. The total number of colonies is indicated at the *bottom* of each image. To the *right* of each photograph is a high magnification image. *Scale bars*, 200 μm. The ratio of AP-positive colonies is shown in *lower panels* after normalization against MeBIO-treated control cells (value = 1). The value shown is the mean ± S.D. of three independent experiments. Significant values are indicated; *, $p < 0.01$; **, $p < 0.03$, in comparison with control. *D–F*, real time PCR analysis of several germ layer markers at 4 days after transfection in the absence of LIF. The results are shown after normalization against MeBIO-treated control cells (value = 1). The values shown are the means ± S.D. of two independent experiments.

pared with MeBIO treatment. This indicates that BIO inhibited differentiation of control cells. In turn, maintenance of *Nanog* expression by autocrine/paracrine Wnt/β-catenin signaling through HS chains is important for the maintenance of pluripotency of mES cells.

DISCUSSION

In this study, we have demonstrated that *EXT1*-deficient mES cells, which express greatly reduced levels of HS chains, proliferate slowly and differentiate spontaneously into the extraembryonic endoderm lineage. Signaling by Wnt and FGF, but not BMP and LIF, was disrupted in *EXT1*-deficient cells. Furthermore, we demonstrated that autocrine/paracrine Wnt/β-catenin signaling through HS chains was necessary for maintenance of Nanog expression, self-renewal, and pluripotency in mES cells even in the presence of LIF/STAT3 and BMP/Smad signaling. Our results indicate that HS chains are important regulators of the extrinsic signaling

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pathways contributing to self-renewal and pluripotency of mES cells.

We designed constructs expressing two different siRNAs targeting *EXT1*. Knockdown of *EXT1* in response to *EXT1-1* siRNA was maintained at ~20% of the level observed in control cells for at least 3 days following selection, even in the absence of puromycin. However, when *EXT1-2* siRNA was used, the level of *EXT1* mRNA recovered to 50–60% of that of control cells 3 days after selection (data not shown). As a result, the effects of *EXT1* knockdown were somewhat weaker in cells transfected with the *EXT1-2* plasmid (Figs. 2–5), reflecting a response that correlated with *EXT1* mRNA levels. These observations strongly support the conclusion that our results reflect the function of HS chains.

In this study, we used a transient knockdown system and found that the efficiency of knockdown decreased daily following the ending of selection. We therefore used puromycin to select for efficient knockdown in cells throughout the experiments described in Fig. 3 and Fig. 6, *D–F*. However, in the self-renewal and proliferation assays (Fig. 2 and Fig. 6*C*), the cells had to be replated at low densities, which rendered them susceptible to puromycin-induced cell death. Thus, puromycin could not be used after replating. These different conditions underlie the apparent experimental disparities such as that between Fig. 2*A*, in which undifferentiated colonies derived from *EXT1*-deficient cells were present at half of the level of control cells, and Fig. 3*A*, in which almost all *EXT1*-deficient cells differentiated.

Several signaling molecules, such as LIF/STAT3, BMP/Smad, Wnt/β-catenin, PI3K/Akt, and Src family members, are required for self-renewal of mES cells (7–12, 38, 39). Until now, it has been believed that extrinsic stimulation by both LIF and serum, including BMP, was sufficient to maintain mES cell self-renewal (4). However, in this study, we showed that self-renewal of mES cells was significantly decreased in response to the reduction in HS chain expression, even when LIF- and serum-mediated signaling were not reduced (Fig. 2 and Fig. 4). As shown in Fig. 5, several Wnts were expressed in mES cells, indicating that autocrine/paracrine Wnts function in mES cells. Indeed, autocrine/paracrine Wnt/β-catenin signaling was actually observed in cells cultured under feeder-free conditions. Furthermore, we showed that Wnt3a binds to the mES cell surface mediated by HS chains, and we demonstrated that autocrine/paracrine Wnt/β-catenin signaling through HS chains is important for maintenance of self-renewal of mES cells (Fig. 5 and Fig. 6). Thus, we propose that maintenance of mES cell self-renewal requires not only LIF plus serum factors but also autocrine/paracrine Wnts.

Reduction of HS chain expression also led to decreased signaling by FGF (Fig. 4). It has been demonstrated that HS chains regulate mouse fibroblast cell proliferation through FGF signaling (41). FGF has been also reported to maintain self-renewal of human ES cells (42), although this effect has yet to be observed in mES cells. We speculate that FGF signaling through HS chains may contribute to the maintenance of self-renewal and proliferation of mES cells, if FGF is present in serum or the conditioned medium. This idea is supported by the fact that HS chains have been shown to play crucial roles in FGF signaling during development (18).

We did not observe a reduction in BMP/Smad signaling in response to reduced short HS chains in mES cells (Fig. 4*B*), although previous reports have indicated that HS chains contribute to signaling by BMP in *Drosophila* and *Xenopus* (18, 43). The specific decrease in signaling by Wnt and FGF, but not by BMP, mediated by short HS chains in *EXT1*-deficient cells may be because of the effects of reduced HS chain length on the ability to accumulate sufficient ligands for their cognate receptors. This is supported by the result that HS chain depletion by heparitinase treatment led to not only further reduction in FGF signaling but also a reduction in BMP/Smad signaling (Fig. 4, *C* and *D*).

IL-6 is a heparin/HS-binding cytokine, and HS chains may regulate paracrine IL-6 signaling (44). Although LIF is an IL-6 family member and might also bind to HS chains, we demonstrated that HS chains are not required for LIF/STAT3 signaling. Neither reduction of HS chains (Fig. 4, *A* and *B*) nor HS chain depletion by heparitinase treatment (Fig. 4, *C* and *D*) had an effect on LIF/STAT3 signaling. Taken together, HS chains contribute to the regulation of several signaling pathways mediated by Wnt, BMP, and FGF but not LIF in mES cells (Fig. 7).

As shown in Fig. 6*C*, the total numbers of colonies scarcely differed between BIO-treated cells and MeBIO-treated cells, and we observed no effect of BIO on mES cell proliferation (data not shown), indicating that BIO treatment had no effect on cell proliferation. As shown in Fig. 2*B*, HS chains contribute to mES cell proliferation. Thus, we suggest that HS chain-mediated signaling pathways that are not influenced by Wnt/β-catenin control mES cell proliferation.

Nanog expression was increased in response to BIO treatment in both control and *EXT1*-deficient cells (Fig. 6*A*). However, the difference in the extent of increase in the expression of *Nanog* mRNA following BIO treatment of control and *EXT1*-deficient cells (Fig. 6*A*) indicates that other signaling pathways contribute to the control of its expression through HS chains. Recently, it has been demonstrated that *Nanog* transcription is regulated by an interaction between Oct3/4 and Sox2 or a novel pluripotential cell-specific Sox element-binding protein (45, 46). As such, the difference in the extent of the increase in the expression of *Nanog* mRNA following BIO treatment of control and *EXT1*-deficient cells may be due to a possible reduction in Oct3/4 and Sox2 or pluripotential cell-specific Sox element-binding protein mediated by unknown signaling through HS chains.

Expression of *Oct3/4* and *LRH-1* mRNA was decreased in *EXT1*-deficient cells treated with MeBIO (Fig. 6*A*), and their expression was not affected by BIO treatment in either control or *EXT1*-deficient cells (Fig. 6*A*). Thus regulation of *Oct3/4* and *LRH-1* expression does not require Wnt/β-catenin signaling through HS chains. LRH-1 has been shown to regulate the expression of *Oct3/4* by binding to its proximal enhancer and promoter (40). LRH-1 has also been shown to play an important role in the regulation of cell proliferation (47). The signaling pathways controlling the expression of LRH-1 remain to be determined. The decrease in *Oct3/4* expression observed in *EXT1*-deficient cells may be depend-

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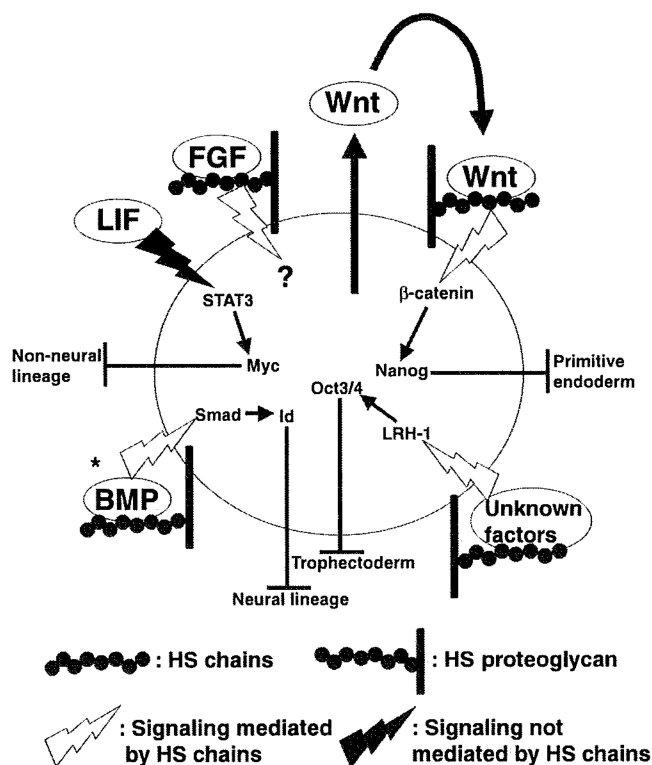

FIGURE 7. **HS chains regulate signaling by extrinsic factors required for the maintenance of self-renewal and pluripotency of mES cells.** HS chains contribute to the regulation of several signaling pathways mediated by extrinsic factors, such as BMP, Wnt, FGF, and unknown factors, but not to LIF and unknown serum factors in mES cells. We demonstrated that autocrine/paracrine Wnt/β-catenin signaling mediated by HS chains regulates Nanog expression and sustains self-renewal by suppression of primitive endodermal differentiation. BMP/Smad signaling is regulated by HS chains (Fig. 4, *C* and *D*) and blocks neural differentiation by induction of Id (11). FGF signaling is also regulated by HS chains (Fig. 4), but the signaling pathway required for maintenance of self-renewal is unknown in mES cells. From the observations made in this study, we suggest that HS chain-dependent signaling by unknown factors regulates mES cell proliferation and Oct3/4 expression. LIF/STAT3 signaling is not regulated by HS chains (Fig. 4) and blocks non-neuronal differentiation by induction of Myc (51). *, BMP/Smad signaling is not decreased by short HS chains (~35 kDa) but is decreased by depletion of HS chains.

ent upon the decrease in LRH-1 expression because of reduced signaling in these unidentified pathways in an HS chain-dependent manner. Although the signaling pathways activated downstream of HS chain expression remain to be determined, those required for LRH-1 expression might regulate the proliferation of mES cells.

In conclusion, we have demonstrated the importance of HS chains for the regulation of self-renewal and pluripotency of mES cells. These results suggest that it is possible to modify self-renewal and differentiation of ES cells by the use of RNAi targeted to genes encoding enzymes required for HS chain synthesis. Signaling by Wnt, FGF, and Notch have been shown to be involved in the regulation of self-renewal of hematopoietic and neural stem cells (48–50). However, the contribution of glycochains to such signaling in these cells has not yet been determined. Modulation of the glycosylation pathways in stem cells will provide the opportunity to engineer stem cells to follow specific differentiation programs that may be of use for the development of cell replacement therapies.

Acknowledgment—We thank Aya Juni for guiding ES cell culture.

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