Lefty1 and *Lefty2* Control the Balance Between Self-Renewal and Pluripotent Differentiation of Mouse Embryonic Stem Cells

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Lefty expression has been recognized as a stemness marker because Lefty is enriched both in undifferentiated embryonic stem cells (ESCs) and in blastocysts. Here, we examined the function of *Lefty1* and *Lefty2* in the maintenance of self-renewal and pluripotency of mouse ESCs (mESCs). Suppression of *Lefty1* or *Lefty2* expression in mESCs did not alter the self-renewal properties of mESCs under nondifferentiating conditions, but suppression of these genes did affect Smad2 phosphorylation and differentiation. *Lefty1* knockdown mESCs showed enhanced phosphorylation of Smad2 and increased differentiation potential, whereas *Lefty2* knockdown mESCs exhibited reduced phosphorylation of Smad2 and enhanced self-renewal in the presence of a differentiation signal. In vivo, teratomas developed from *Lefty2* knockdown mESCs contained massive expansions of immature neuroepithelium, a marker of malignant teratomas. Taken together, these results suggest that optimal expression of *Lefty1* and *Lefty2* is critical for the balanced differentiation of mESCs into three germ layers.

Introduction

Transforming growth factor (TGF)-β family members are enriched in embryonic stem cells (ESCs), suggesting that these proteins are part of critical signaling pathways that maintain the stemness and pluripotency of these cells [1]. Among inhibitors of TGF-β ligands, only Lefty is enriched both in undifferentiated ESCs and in blastocysts, which indicates that Lefty expression is a marker of stemness [2–4]. Lefty is also highly expressed in the inner cell mass and trophoectoderm [2]. In mouse ESCs (mESCs), Lefty expression is regulated by the binding of a cooperative transcriptional complex composed of *Klf4*, *Oct4*, and *Sox2* to the proximal element of the *Lefty1* promoter [5]. However, unlike ESC self-renewal genes such as *Oct4*, Lefty expression is not quenched upon differentiation of ESCs. When leukemia inhibitory factor (LIF) is removed from the culture medium of mESCs, the expression of Lefty increases within 48 h of cytokine withdrawal [6]. Similarly, differentiation of ESCs to an embryoid body (EB) leads to increased expression of Lefty [7]. Retinoic acid (RA)–mediated differentiation of ESCs also leads to an increase in Lefty expression in mouse embryonal carcinoma cells [8]. Therefore, Lefty might be important both for the maintenance of self-renewal and the exit from this state that leads to the differentiation of ESCs.

In both humans and mice, the Lefty genes have been localized to chromosome 1 and the locus contains two genes—*Lefty1* and *Lefty2*—with the same transcriptional orientation, and one pseudogene—*Lefty3*—that has a reverse transcrip-

tional orientation [9,10]. *Lefty1* has 91% sequence identity and shares 331 amino acids with *Lefty2*, indicating that *Lefty1* and *Lefty2* are closely related to each other. *Lefty1* and *Lefty2* both block Nodal signaling by binding Nodal and its EGF-CFC coreceptors, such as TDGF-1/Cripto. These interactions prevent the assembly of an active Nodal/Activin receptor complex [11,12].

Even though several findings suggest that TGF-β signaling is required for the maintenance of pluripotency of ESCs [13], the precise role of *Lefty1* or *Lefty2* in ESCs remains to be elucidated. Recently, our research group reported that the *Lefty1*-Nodal-Smad2 pathway regulated by *Tcea3* is an innate program to determine cell fate choices between self-replication and commitment to differentiation [14]. Here, we studied the function of *Lefty* isoforms in relation to pluripotency by examining the effect of *Lefty1* or *Lefty2* suppression on the self-renewal and differentiation of mESCs. Suppression of *Lefty1* and *Lefty2* produced opposing effects on the differentiation of mESCs. *Lefty1* knockdown mESCs (*Lefty1* KD) showed enhanced phosphorylation of Smad2 and enhanced differentiation potential, whereas *Lefty2* knockdown mESCs (*Lefty2* KD) exhibited reduced phosphorylation of Smad2, which might be the result of enhanced expression of *Lefty1*. In addition, *Lefty2* KD mESCs showed enhanced self-renewal and reduced differentiation in response to a differentiation signal. An in vivo teratoma assay showed that *Lefty2* KD mESCs formed more malignant tumors that had higher expression of self-renewal factors, such as *Oct4* and *Sox2*. These results suggest that

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balanced expression of *Lefty1* and *Lefty2* is critical to maintain the pluripotency of mESCs and that optimal expression of *Lefty2* is essential to inhibit carcinogenesis of ESCs.

Materials and Methods

Cell culture, EB formation, and in vitro differentiation of mESCs

J1 mESCs (Cat. No. SCRC-1010) were purchased from ATCC (www.atcc.org) and maintained as described previously [15]. The mESCs were cultured in ESC medium [Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal calf serum (HyClone), 0.1 mM 2-mercaptoethanol (Sigma), 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM glutamine (Gibco), and 1,000 U/mL LIF (Chemicon)]. To induce mESC differentiation, mESCs were cultured in LIF-deficient ESC medium containing 100 nM all-*trans* RA. To form EBs, mESCs were trypsinized to achieve a single-cell suspension and, subsequently, cultured on uncoated Petri dishes in ESC medium without LIF. The medium was changed every 2 days for mESC culture or differentiation. Four days after primary EB formation, EBs were collected and dissociated into single cells by trypsinization and trituration. These EB cells were replated into ESC medium without LIF and the efficiency of secondary EB production was assessed after 10 days, to determine the proportion of undifferentiated mESCs present in the primary EBs. Activin-induced mesoendoderm differentiation was performed as previously described [16]. Briefly, ESCs were cultured as a monolayer in gelatinized feeder-free six-well plates with an initial plating density of 1×10^5 cells/well and the time when 25 ng/mL of Activin was added was counted as day 0. The medium was composed of a 1:1 mixture of DMEM/F12 (Invitrogen) supplemented with N2 supplement (Stem Cell Technologies) and NeuralBasal medium (Invitrogen) supplemented with B27 supplement (Stem Cell Technologies) and β-mercaptoethanol. Cells were harvested at day 4 for gene expression analysis.

Genetic modification of mESCs

shRNA plasmids that target mouse *Lefty1* and *Lefty2* were purchased (RMM4534-EG13590 and RMM4534-EG320202; Open Biosystems) to generate knockdown cell lines of mESCs. Plasmids were transfected into mESCs with Lipofectamine 2000 (Invitrogen) and stably transfected lines were established according to the manufacturer's instructions. Five *Lefty1* KD and five *Lefty2* KD cell lines were established, and two different cell lines of each knockdown were used to study the effect of suppression of *Lefty1* or *Lefty2* in mESCs.

Alkaline phosphatase activity

Alkaline phosphatase activity was measured using the AnaSpec kit (No. 71230; AnaSpec, www.anaspec.com) according to the manufacturer's instructions.

Cell cycle analysis by flow cytometry

Samples (1×10^6 cells) were washed with 0.5 mL phosphate-buffered saline (PBS) and fixed with ice-cold 70% (v/v) ethanol at 4°C for at least 1 h. After washing in PBS again, cells were incubated with staining buffer containing 50 µg/mL propidium iodide (Sigma), 0.2 mg/mL RNase A (Sigma), and 0.1% (v/v) Triton X-100 in PBS in the dark for 40 min at 37°C. The DNA content was analyzed using an FACSCalibur flow cytometer (BD Biosciences) and the data were analyzed using Cell Quest software (BD Biosciences).

RNA extraction and real-time reverse transcriptase (RT)-PCR

Total RNA from mESCs and teratomas was extracted using TRIzol (Invitrogen), and 2–5 µg of total RNA was reverse-transcribed using the SuperScriptII™ First-Strand Synthesis System (Invitrogen) according to the manufacturer's instructions. The cDNA was treated with 2 U of RNase H (Invitrogen) for 20 min at 37°C. Real-time RT-PCR was carried out using cDNAs with the Quantitect SYBR Green PCR kit (Qiagen). Reactions were carried out in triplicate using an Exicycler™ 96 (Bioneer). For quantification, target genes were normalized against glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*). PCR primers used in this study are listed in Supplementary Table S1 (Supplementary Data are available online at www.liebertpub.com/scd).

Immunoblot analysis

For immunoblotting assays, cells were washed twice with cold PBS, lysed with tissue lysis buffer [20 mM Tris-base (pH 7.4), 137 mM NaCl, 2 mM EDTA, 1% Triton X-100, 25 mM β-glycerophosphate, 2 mM sodium pyrophosphate, 10% glycerol, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 1 mM benzamidine], and clarified by centrifugation at 12,000 *g* for 10 min. Whole-cell extracts were prepared and 20–50 µg of proteins was resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes (Perkin Elmer Life Sciences), and probed using antibodies against Oct4 (sc-9081; Santa Cruz), Sox2 (sc-20088; Santa Cruz), pStat3 (Tyr-705) (No. 9131; Cell Signaling Technology), Stat3 (sc-482; Santa Cruz), pSmad2 (No. 3101; Cell Signaling Technology), Smad2/3 (sc-8332; Santa Cruz), or β-actin (sc-47778; Santa Cruz). Immunoreactivity was detected by enhanced chemiluminescence (ECL; Amersham). For quantitative analysis, the mean density of each band was measured using Multi Gauge V3.0 software.

Teratoma formation

For teratoma formation assays, cells were trypsinized, and 5×10^5 cells were suspended in a DMEM/Matrigel solution [BD Biosciences, Inc.; 1:1 ratio (v/v)]. The cell/Matrigel suspension was then injected subcutaneously into NOD/SCID mice (Charles River Laboratories). Teratoma formation of Lefty1 KD and Lefty2 KD was examined at 6 and 4 weeks after injection, respectively. The experiments were reviewed and approved by the Institutional Animal Care and Use Committee of CHA University. All procedures were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH publication No. 85-23, revised 1996).

Statistical analysis

Graphical data are presented as mean ± SD. Each experiment was performed at least three times and subjected to

statistical analysis. Significant differences between two groups were determined using Student's *t*-test. $P < 0.05$ was considered significant. $P < 0.05$ and $P < 0.01$ were marked as ** and *. Statistical analysis was performed using the SAS statistical package v.9.13 (SAS, Inc.).

Results

Lefty1 *critically controls the differentiation potential of mESCs*

To study the role of *Lefty1* and *Lefty2* in mESCs, we first generated mESC lines that stably expressed shRNA for *Lefty1* or *Lefty2* transcripts. The relative transcript levels of *Lefty1* in the *Lefty1* KD were <50% of wild type (WT) (Fig. 1A). Because Lefty inhibits phosphorylation of Smad2/3 by blocking Nodal/Activin signaling, we examined the phosphorylation of Smad2 in *Lefty1* KD cell lines. As expected, knockdown of *Lefty1* expression resulted in an increase in Smad2 phosphorylation in ESCs (Fig. 1B). The expression of self-renewal factors, such as Oct3/4 and Sox2, was not affected by the suppression of *Lefty1* (Fig. 1C). In addition, the level of alkaline phosphatase activity in *Lefty1* KD cells was comparable with that in the WT, indicating that changes in the level of *Lefty1* expression did not affect the self-renewal of mESCs (Fig. 1D). The cell proliferation and cell cycle profiles of *Lefty1* KD cells were similar to those of WT cells (Fig. 1E, F). However, the efficiency of secondary EB formation by

Lefty1 KD cells was much lower than that of WT cells (Fig. 1G). Secondary EB formation reflects the ability of mESCs to maintain their self-renewal capacity [17]. Previously, we reported that *Lefty1*-Nodal-pSmad2 signals regulated by *Tcea3* form a critical pathway that regulates cell fate choices between self-renewal and commitment to differentiation [14]. As *Lefty1* is a negative regulator of Nodal signaling and Smad2-mediated Activin/Nodal signaling is required for proper differentiation of mESCs toward the mesoendoderm lineages [16], we hypothesized that suppression of *Lefty1* expression would facilitate differentiation of mESCs. As expected, upon removal of LIF and addition of RA, *Lefty1* KD cells rapidly developed epithelial-like outgrowths by day 2 whereas WT mESCs still maintained ESC-like colonies (Fig. 2A). Consistent with the differentiating cell morphology, the expression of lineage markers was markedly increased in differentiating *Lefty1* KD cells compared with that in the WT (Supplementary Fig. S1). Despite the facilitated differentiation of *Lefty1* KD cells, cell proliferation and the cell cycle profiles of *Lefty1* KD cells were similar to those of the WT (Supplementary Fig. S2A, B). Smad2 phosphorylation in the differentiating *Lefty1* KD cells was maintained at a higher level than that in WT cells, whereas Sox2 levels rapidly decreased in differentiating *Lefty1* KD cells (Fig. 2B). When a *Lefty1*-expressing plasmid was transiently introduced, the differentiation properties of *Lefty1* KD cells became similar to WT (Fig. 2C). Consistent with the in vitro differentiation properties, *Lefty1* KD cells developed
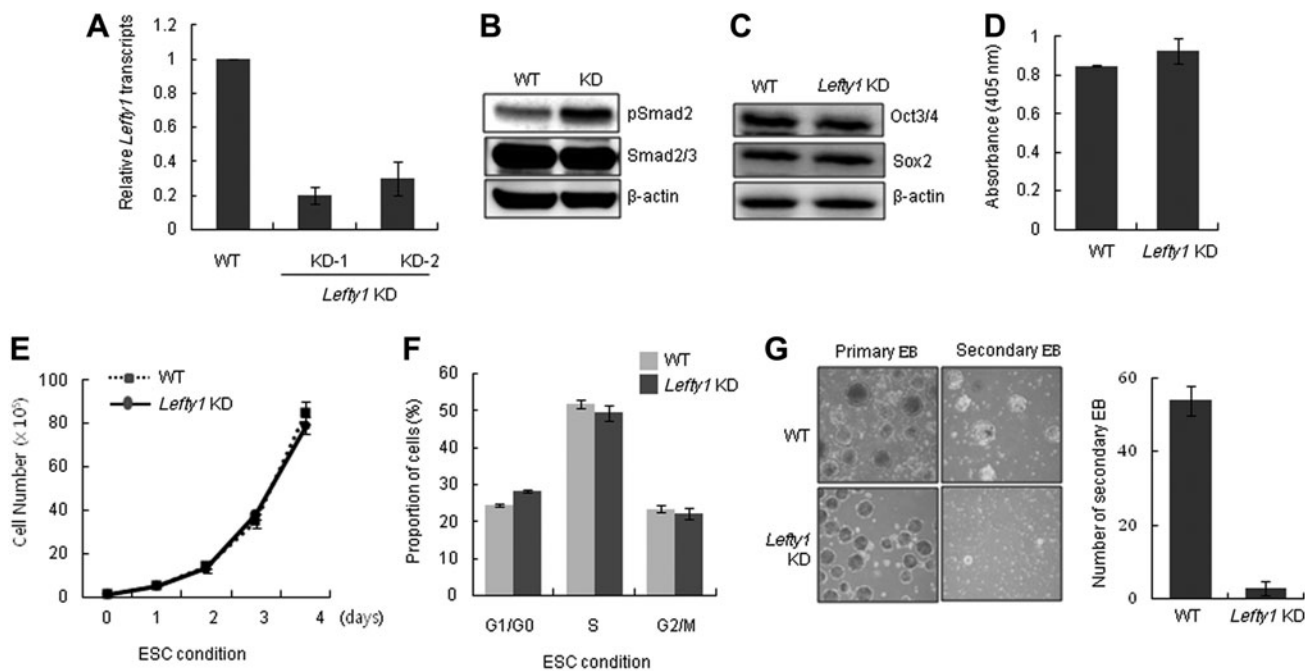

FIG. 1. Suppression of *Lefty1* expression does not affect the self-renewal of mESCs. **(A)** Expression level of *Lefty1* in two *Lefty1* KD (KD-1 and KD-2) cell lines was analyzed by real-time reverse transcriptase (RT)-PCR. **(B)** Phospho-Smad2 of *Lefty1* KD cells under self-renewal culture conditions was analyzed by immunoblot analysis. **(C)** Expression of Oct4 and Sox2 by *Lefty1* KD cells under ESC culture conditions was analyzed by immunoblot analysis. **(D)** Alkaline phosphatase activity of *Lefty1* KD cells was compared with that in WT cells. **(E)** The proliferation of *Lefty1* KD cells was compared with that of WT cells at the indicated times. **(F)** The cell cycle distribution of *Lefty1* KD cells was compared with that of WT cells by flow cytometry. **(G)** Primary EBs and secondary EBs formed by *Lefty1* KD and WT cells were examined by light microscopy after 4 and 10 days in culture, respectively (*left panel*). The number of secondary EBs was compared between WT and *Lefty2* KD mESCs (*right panel*). All values are mean ± SD from at least three independent experiments. *Lefty1* KD, stable *Lefty1*-knocked-down mESC line; WT, wild-type mESCs; EB, embryoid body; mESCs, mouse embryonic stem cells.
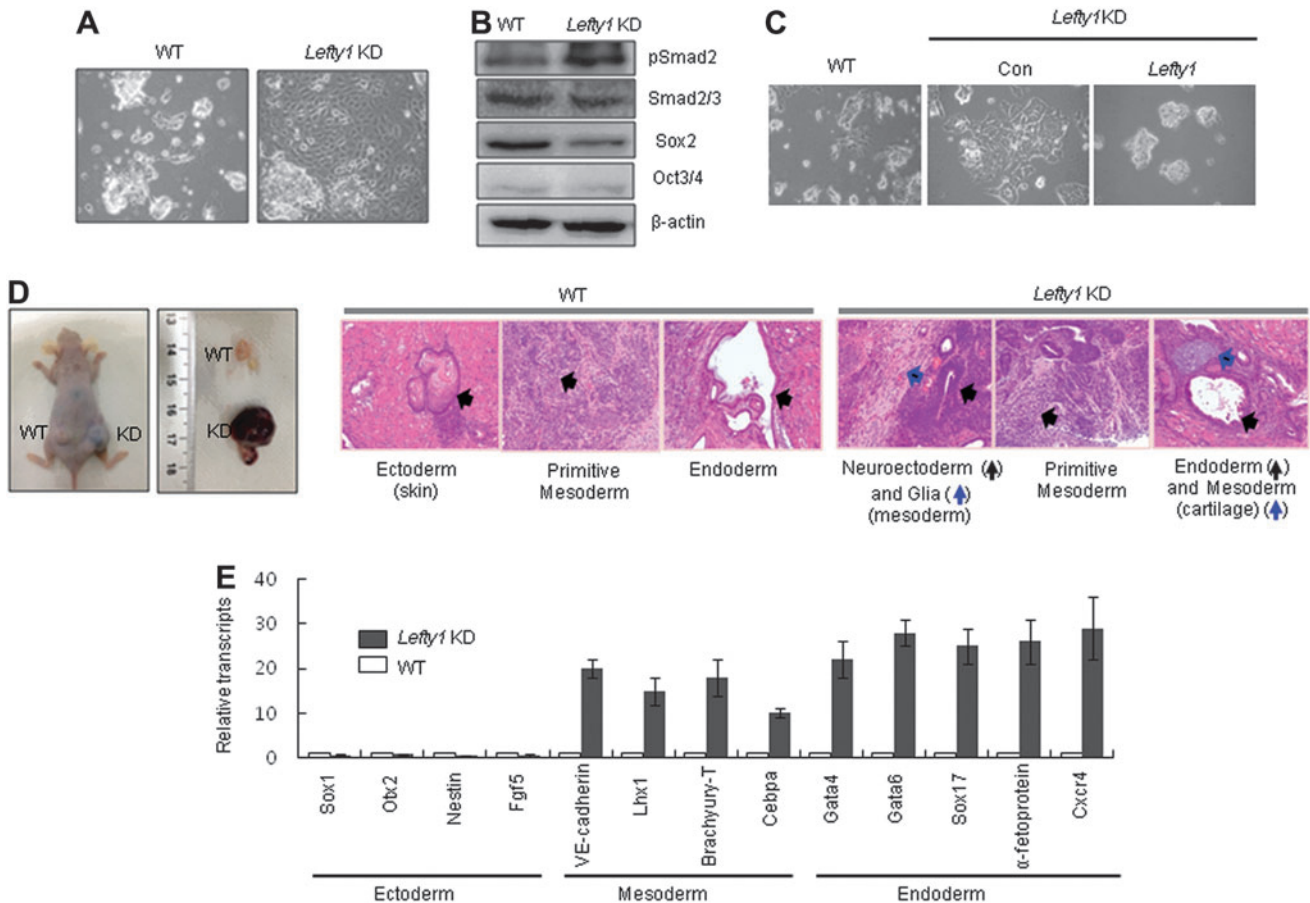
FIG. 2. Suppression of *Lefty1* expression enhances differentiation potential of mESCs. **(A)** *Lefty1* KD cells spontaneously differentiate when leukemia inhibitory factor (LIF) is removed and RA is added for 3 days. Cell morphology was examined by light microscopy. **(B)** Expression of phospho-Smad2 and self-renewal markers, including Oct4 and Sox2, by differentiating *Lefty1* KD cells was analyzed by immunoblotting. **(C)** *Lefty1* KD cells were transfected with control (Con) or a *Lefty1* expression plasmid and then spontaneously differentiated for 3 days. Differentiating cells were examined by light micros-copy. **(D)** *Lefty1* KD and control mESCs were injected into NOD/SCID mice and the size of the teratomas was examined 4 weeks after injection. Teratomas were stained with hematoxylin-eosin (H&E) and the skin, primitive mesoderm, glia, and cartilage in the teratomas were identified by examination under a light microscope. **(E)** Lineage-specific marker expression in *Lefty1* KD teratomas was compared with that of WT by real-time RT-PCR. All values are mean ± SD from at least three independent experiments. *Lefty1* KD, stable *Lefty1*-knocked-down mESC line; WT, wild-type mESCs; RA, retinoic acid.

teratomas more rapidly in vivo than WT (Fig. 2D, left panel). When examined by histological staining, teratomas harvested 4 weeks after transplantation of *Lefty1* KD cells showed well-developed and differentiated glia (mesoderm) and cartilage (mesoderm), indicating that *Lefty1* KD cells had a tendency to differentiate into cells of the mesodermal lineage (Fig. 2D, right panel).

Markedly enhanced expression of mesoderm and endo-derm markers in 4-week-old *Lefty1* KD teratomas suggested that larger teratoma formation was an in vivo indicator of differentiation potential (Fig. 2E). To investigate whether the activation of TGF-β signaling in the *Lefty1* KD cells leads to the increased differentiation of mESCs, we examined the effects of a chemical inhibitor of TGF-β signaling upon the differentiation of *Lefty1* KD cells. Treatment with SB431542 compromised the morphology of rapidly differentiating mESC colonies and the increased expression of lineage markers in the differentiating *Lefty1* KD cells (Supplementary Fig. S3A, B). These results indicate that *Lefty1* critically con-

trols the differentiation potential of mESCs via a TGF-β sig-naling pathway.

Suppression of Lefty2 *impairs differentiation of mESCs*

We next examined the function of *Lefty2* in mESCs by generating *Lefty2* KD cell lines using an shRNA targeting *Lefty2* (Fig. 3A). Suppression of *Lefty2* expression did not affect the expression of self-renewal markers, such as phos-phorylated Stat3 (p-Stat3), Sox2, and Oct4 (Fig. 3B). The cell proliferation and cell cycle profiles of the *Lefty2* KD cell populations were similar to those of the WT both under self-renewal and differentiation culture conditions (Fig. 3C and Supplementary Fig. S4). In addition, the alkaline phospha-tase activity of *Lefty2* KD cells was similar to that of WT cells (Fig. 3D). These results suggest that alteration of *Lefty2* ex-pression does not have any effect on the self-renewal of mESCs. We next investigated whether *Lefty2* has a similar
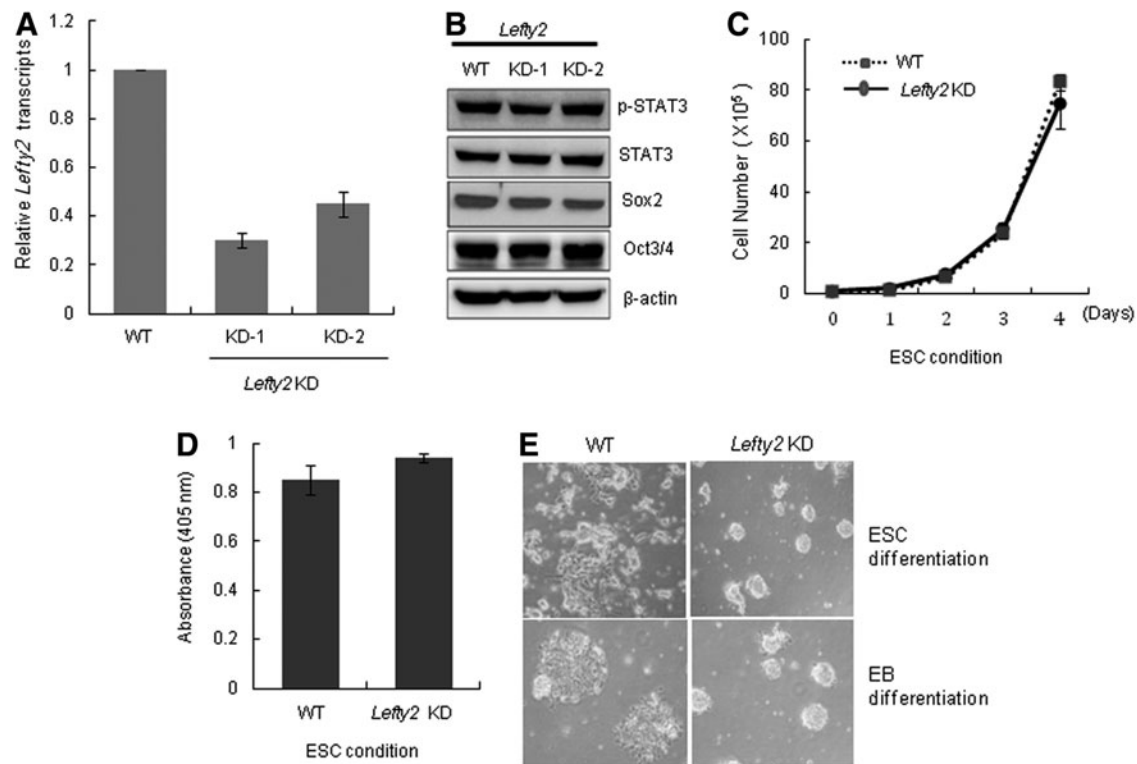
FIG. 3. *Lefty2* KD mESCs have defective differentiation properties. **(A)** Expression level of *Lefty2* in two *Lefty2* KD (KD-1 and KD-2) cell lines was analyzed by real-time RT-PCR. **(B)** The expression of phospho-Stat3, Sox2, and Oct3/4 by *Lefty2* KD cells under ESC culture conditions was analyzed by immunoblotting. **(C)** Proliferation of *Lefty2* KD cells in ESCs was compared with that of WT cells at the indicated times. **(D)** Alkaline phosphatase activity of *Lefty2* KD cells was compared with that in the WT. **(E)** *Lefty2* KD cells spontaneously differentiated (*upper*) or differentiated into the mesoendoderm lineage (*bottom*) for 3 days and cell morphology was examined by light microscopy. All values are mean±SD from at least three independent experiments. *Lefty2* KD, stable *Lefty2*-knocked-down mESC line; WT, wild-type mESCs.

function to *Lefty1* in regulating pluripotent differentiation of mESCs. In contrast to *Lefty1* KD, *Lefty2* KD cells maintained undifferentiated colony morphology under RA-induced differentiation conditions (Fig. 3E, upper panel). To confirm the effect of *Lefty2* suppression on the differentiation of mESCs, we cultured mESCs under mesoendoderm differentiation conditions. Consistent with the RA-induced spontaneous differentiation pattern, *Lefty2* KD cells did not respond to differentiation stimuli (Fig. 3E, lower panel). Based on these results, we concluded that suppression of *Lefty2* significantly impairs the pluripotent differentiation potential of mESCs.

Suppression of Lefty2 results in enhanced expression of Lefty1

Because *Lefty2* functions as a negative regulator of Nodal signaling and active Nodal/Activin signaling induces phosphorylation of the effectors Smad2/3 [18], we investigated whether suppression of *Lefty2* leads to phosphorylation of Smad2. Surprisingly, the levels of phosphorylated Smad2 (p-Smad2) in *Lefty2* KD cells were lower than those in the WT, and were further reduced when cells were differentiated for 3 days under RA-mediated differentiation conditions (Fig. 4A). This result suggests that Smad2-mediated Activin/Nodal signaling, which is required for the proper

differentiation of mESCs toward mesoendoderm lineages, was impaired in the *Lefty2* KD cell lines. To confirm that the defective differentiation phenotype of *Lefty2* KD cell lines was caused by suppression of *Lefty2* expression, we rescued *Lefty2* expression by introducing a *Lefty2* expression plasmid into *Lefty2* KD and then examined whether increased *Lefty2* expression could rescue the defective differentiation of *Lefty2* KD. The differentiation pattern of *Lefty2* KD became similar to WT as a result of re-expression of *Lefty2* (Fig. 4B). When *Lefty2* was re-expressed in *Lefty2* KD mESCs, phosphorylation of Smad2 increased, consistent with our hypothesis that the low level of p-Smad2 in the differentiating *Lefty2* KD mESCs was indeed caused by suppression of *Lefty2* expression (Fig. 4C).

To understand the underlying mechanism of dephosphorylation of Smad2 and the impaired differentiation of *Lefty2* KD cells, we examined whether the inhibition of *Lefty2* expression in mESCs results in the suppression of Nodal expression. However, Nodal expression was increased in *Lefty2* KD cells both in ESC culture and in differentiation conditions (Fig. 4D). We then analyzed the expression level of *Lefty1*, another negative regulator of Nodal-mediated Smad2 phosphorylation. Interestingly, the expression of *Lefty1* was markedly increased in both ESCs and differentiating *Lefty2* KD cells (Fig. 4E). These results suggest that abnormal overexpression of *Lefty1* is the main cause of reduced
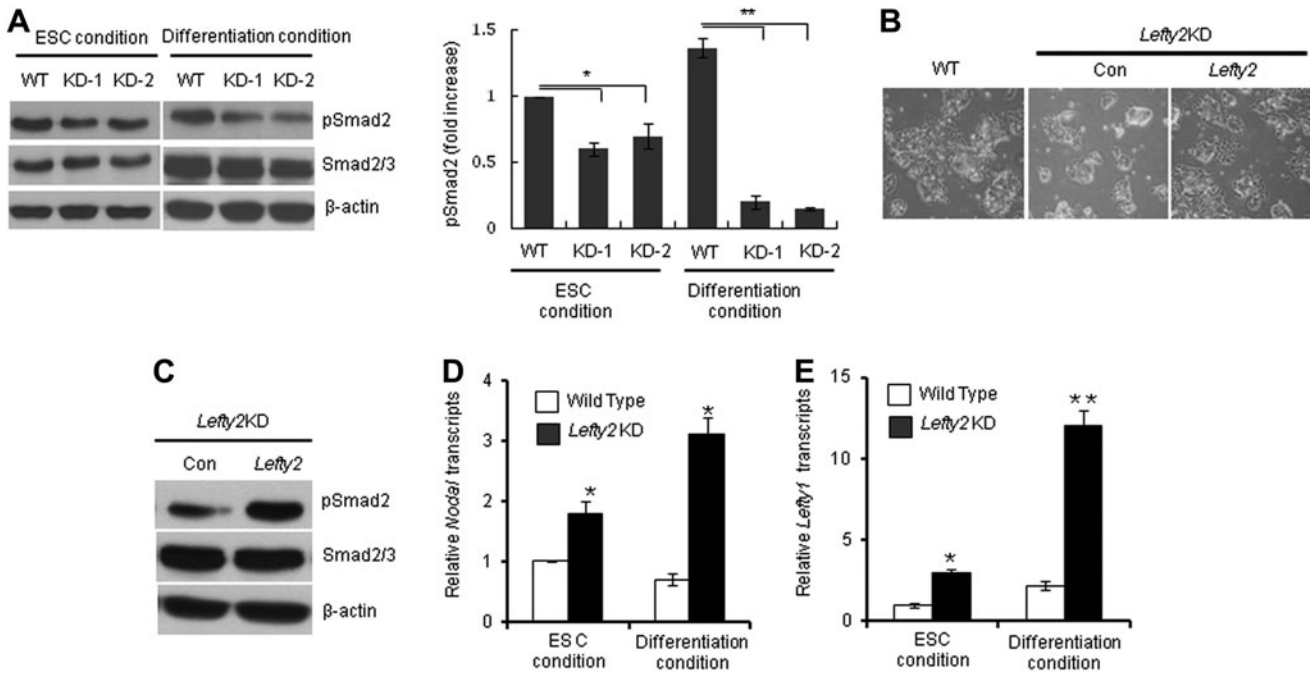
FIG. 4. Smad2 phosphorylation and *Lefty1* expression in *Lefty2* KD cells. **(A)** Phospho-Smad2 in two *Lefty2* KD mESC lines under self-renewal or differentiation culture conditions was analyzed by immunoblot analysis (*left panel*). The band intensity of p-Smad2 was quantified by normalization against β-actin (*right panel*). Cells were differentiated by removing LIF and adding RA for 3 days (RA-d3). **(B)** *Lefty2* KD was transfected with a control (Con) or a *Lefty2* expression plasmid and then spontaneously differentiated for 3 days. Differentiating cells were examined by light microscopy. **(C)** *Lefty2* KD mESCs transfected with a control (Con) or a *Lefty2* expression plasmid were analyzed for phospho-Smad2 levels by immunoblot analysis. **(D)** Nodal expression in *Lefty2* KD and control ESCs was analyzed by real-time RT-PCR. **(E)** *Lefty1* expression in *Lefty2* KD and control ESCs was analyzed by real-time RT-PCR. All values are mean ± SD from at least three independent experiments. *$P < 0.05$ and **$P < 0.01$ based on Student's *t*-test analysis. *Lefty2*KD, stable *Lefty2*-knocked-down mESC line; Con, *Lefty2*KD transfected with control plasmid.

phosphorylation of Smad2 and the impaired differentiation of *Lefty2* KD cells. The inhibitory effect of *Lefty1* overexpression on the differentiation of ESCs was further supported by the observation that transient transfection of a *Lefty1*-expressing plasmid inhibited RA-mediated differentiation of mESCs (Supplementary Fig. S5).

Self-renewal is enhanced in the differentiating Lefty2 *KD mESCs*

To further investigate the mechanism of impaired differentiation of *Lefty2* KD cells, we analyzed the expression of self-renewal and differentiation markers in the differentiating *Lefty2* KD mESCs. Unexpectedly, both self-renewal and differentiation marker genes were highly expressed in differentiating *Lefty2* KD cells (Fig. 5A). This result indicates that the expression of self-renewal genes in *Lefty2* KD mESCs was increased upon exposure to differentiation stimuli, which could result in the enhancement of self-renewal of mESCs under differentiation conditions. Immunoblot analysis of self-renewal markers, including phospho-Stat3, Sox2, and Oct3/4, further supports the possibility that self-renewal of *Lefty2* KD was not decreased during RA-mediated differentiation (Fig. 5B). To confirm that the expression of self-renewal genes in differentiating *Lefty2* KD was caused by the suppression of *Lefty2* expression, we analyzed Oct4 and Sox2 expression levels in differentiating *Lefty2* KD that were

transfected with a *Lefty2*-expressing plasmid. The expression of Oct4 and Sox2 in differentiating *Lefty2* KD cells was decreased to that of WT when *Lefty2* expression was rescued (Fig. 5C). The increase in secondary EB formation by *Lefty2* KD cells further supports the hypothesis that self-renewal of *Lefty2* KD is enhanced under differentiating culture condition (Fig. 5D).

Lefty2 *KD teratomas show a massive expansion of immature malignant tissue*

We next analyzed the impact of *Lefty2* on the differentiation of mESCs in xenografts. Similar to *Lefty1* KD cells, *Lefty2* KD cells showed an obvious increase in teratoma growth compared with WT mESCs (Fig. 6A). Because teratomas are an in vivo indicator of pluripotency and in vitro differentiation properties, the knockdown of *Lefty2* suggested that *Lefty2* suppression leads to a defect in the pluripotent differentiation of mESCs. The development of larger teratomas from *Lefty2* KD cells was an unexpected result. As teratomas generated from mESCs contain a small proportion of malignant, undifferentiated carcinoma tissue [19], we hypothesized that the enlarged size of *Lefty2* KD teratomas is due to a large proportion of malignant tissue. Unlike other epithelial cells (eg, squamous epithelial cells or gut-epithelium), immature neuroepithelium that resembles a neural tube was embedded in the glial fibrillary background. The areas of
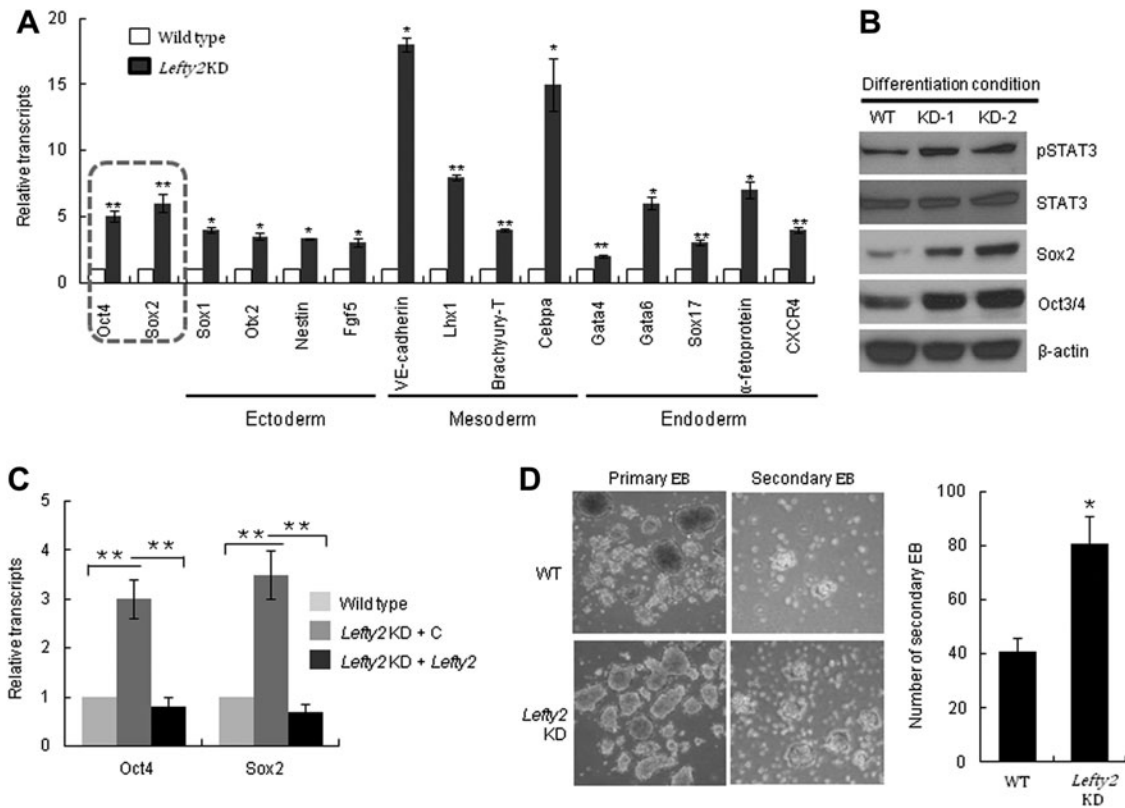
FIG. 5. Self-renewal is not decreased in the differentiating *Lefty2* KD cells. **(A)** *Lefty2* KD cells were spontaneously differentiated for 3 days and expression of the indicated genes was analyzed by real-time RT-PCR. **(B)** *Lefty2* KD cells were spontaneously differentiated for 3 days and expression of phospho-Stat3, Sox2, and Oct4 was analyzed by immunoblot. **(C)** Oct4 and Sox2 expression in differentiating *Lefty2* KD mESCs transfected with a control or a *Lefty2* expression plasmid was compared with differentiating WT mESCs by real-time RT-PCR. mESCs were spontaneously differentiated for 3 days. **(D)** Primary and secondary EBs were formed for 4 days and examined by light microscopy (*left panel*). The number of secondary EBs was compared between WT and *Lefty2* KD mESCs (*right panel*). All values are mean ± SD from at least three independent experiments. *$P < 0.05$ and **$P < 0.01$ based on Student's *t*-test analysis. *Lefty2* KD, stable *Lefty2*-knocked-down mESC line; WT, wild-type mESCs.

immature neuroepithelium were composed of stratified co-lumnar cells that formed rosettes. Fine eosinophilic glial fibrils of neuroglia without a collagen matrix provided a supporting framework for the neuroepithelium, which was confirmed by immunohistochemistry for glial fibrillary acidic protein (data not shown).

In teratomas formed by *Lefty2* KD cells, massive numbers of immature neuroepithelial cells, which represent malignant immature teratogenic cells, were present and the proportion of mature neuroglia was significantly reduced (Fig. 6B upper panel, C). Cartilage and bone tissues were easily detected by their blue-gray matrix or dense calcification, as well as by the presence of lacunar chondrocytes and bony osteocytes. Mesoderm-derived cartilage and/or bone were highly developed in *Lefty2* KD teratomas compared with WT (Fig. 6B lower panel, C). High expression levels of the pluripotency genes Oct4, Sox2, Nanog, and Klf4 in the bulk teratoma tissues derived from *Lefty2* KD cells suggest that prolonged self-renewal might be the main cause of malignant and immature teratoma development (Fig. 6D). Together, these data indicate that suppression of *Lefty2* leads to the malignant transformation of differentiating mESCs.

Discussion

The importance of Activin/Nodal signaling in the earliest cell fate decisions during embryogenesis has been well recognized. Nodal induces mesoderm and endoderm, patterns the nervous system, and determines left-right (L-R) asymmetry in vertebrates. Smad2 mediates Activin/Nodal signaling functions to orchestrate mesoendoderm lineage commitment of mESCs through direct modulation of the expression of corresponding developmental regulators [18]. Therefore, Lefty is a critical regulator of balanced lineage differentiation of cell fate determinants of ESCs. The present study revealed that *Lefty1* functions to regulate mesoendoderm lineage differentiation commitments in response to external differentiation signals. However, in sharp contrast to the differentiation properties of *Lefty1* KD, self-renewal of *Lefty2* KD was not decreased under differentiation culture medium. *Lefty1* is abnormally overexpressed and Smad2 is dephosphorylated in the differentiating *Lefty2* KD, implying that proper expression of *Lefty2* is crucial to maintain optimal *Lefty1* and phospho-Smad2 levels in the differentiating mESCs. In addition, Oct4 and Sox2 are highly expressed in the differentiating *Lefty2* KD. The *Lefty1*
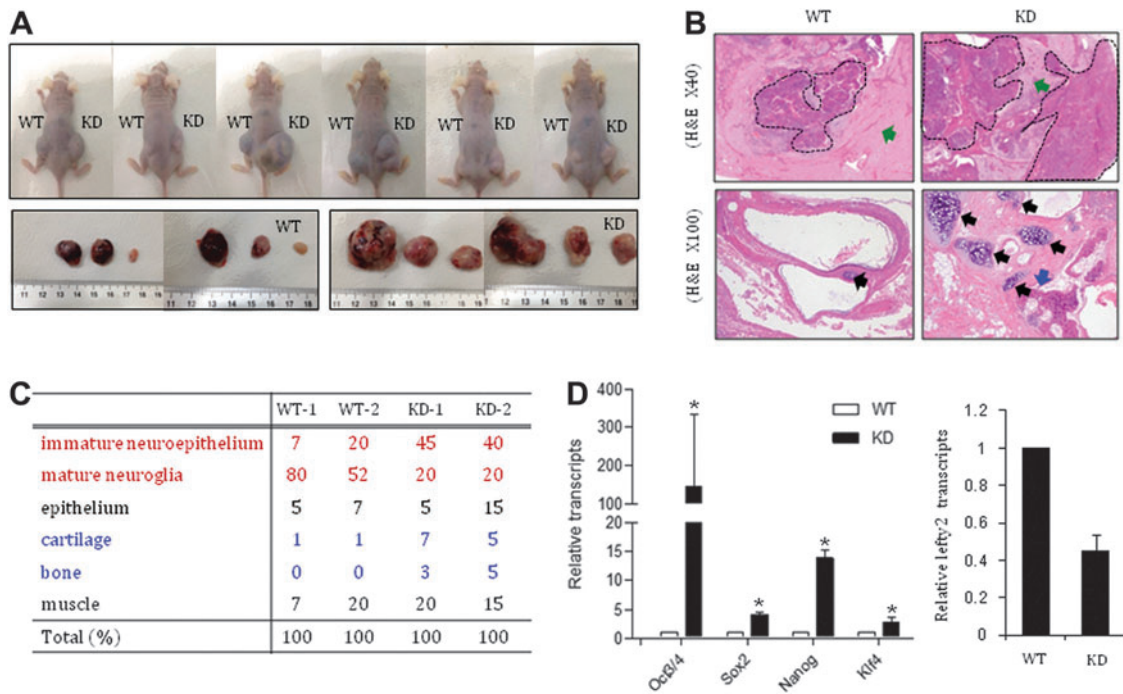
FIG. 6. *Lefty2* KD cells develop immature malignant teratomas. **(A)** *Lefty2* KD and control mESCs were injected into NOD/SCID mice and teratoma development was examined. The size of the teratomas was examined 6 weeks after injection. **(B)** H&E staining and data analysis of teratoma sections were performed by a pathologist. Immature neuroepithelium is indicated by *dotted lines* and mature neuroglia is indicated by *green arrows* (*upper panel*). Cartilage and bone in the teratoma are indicated by *black* and *blue arrows*, respectively (*lower panel*). **(C)** Quantification of immature neuroepithelium, mature neuroglia, epithelium, cartilage, bone, and muscle was performed using serially sectioned teratomas of two WT and two *Lefty2* KD teratomas. **(D)** Oct4, Sox2, Nanog, Klf4, and Lefty2 expression in the teratoma tissue was analyzed by real-time RT-PCR. All values are mean ± SD from at least three independent experiments. *$P < 0.05$ based on Student's *t*-test analysis. KD, stable *Lefty2*-knocked-down mESC line; WT, wild-type mESCs.

promoter contains an ESC-specific enhancer that contains binding sites for Oct4 and Sox2 [5], indicating that overexpression of *Lefty1* in the differentiating *Lefty2* KD is mainly caused by enhanced expression of Oct4 and Sox2. Similar to *Lefty2* KD cells, phospho-Smad2 levels in mESCs that overexpress *Lefty1* (*Lefty1* OE) were lower than those in WT cells (Supplementary Fig. S6A). However, *Lefty1* OE cells developed smaller teratomas, which showed impaired differentiation of the three germ layers (Supplementary Fig. S6B). This result is opposite to that obtained for *Lefty2* KD cells (Fig. 6A). Consistent with the teratoma size, the expression of several lineage markers in *Lefty1* OE cells was lower than that in WT cells (Supplementary Fig. S6C). These results suggest that the Smad2 dephosphorylation in *Lefty2* KD cells was caused by the activation of *Lefty1*, whereas the development of malignant and immature teratomas from *Lefty2* KD cells was caused by a distinct mechanism, such as the increased self-renewal properties of *Lefty2* KD mESCs under differentiation conditions. Contrary to the results obtained with *Lefty2* KD cells, the expression of Nodal or *Lefty2* was not altered in *Lefty1* KD cells (Supplementary Fig. S7). This result implies that Nodal signaling is not regulated by reciprocal interactions of *Lefty1* and *Lefty2*, at least in mESCs.

An in vivo teratoma assay revealed that *Lefty2* KD mESCs formed enlarged teratomas with an enormous expansion of immature tissues. Recent studies demonstrated that LeftyA in conditioned medium from human liver stem cells (MSCs) has antitumor activity [20]. It would be important to elucidate whether *Lefty2* has tumor-suppressive activity that could inhibit the malignant transformation of ESCs by repressing self-renewal properties in the differentiating mESCs. It is interesting to note that cartilage and bone were more developed in *Lefty2* KD teratomas compared with WT. The reinforced differentiation of *Lefty2* KD mESCs, at least into mesodermal lineages, was further supported by the enhanced expression of differentiation marker genes in the differentiating *Lefty2* KD cells in vitro (Fig. 5A). This result indicates that suppression of *Lefty2* also enhances the mesodermal differentiation potential of *Lefty2* KD as well as the malignancy of mESCs. In addition, these results also suggest that enhanced self-renewal in the differentiating mESCs could transform mESCs into malignant carcinoma tissues regardless of the activation of a commitment to differentiation.

Contrary to *Lefty2* KD, *Lefty1* KD mESCs exhibited enhanced phospho-Smad2 levels both in self-renewal and in differentiating cells. In addition, suppression of *Lefty1* facilitates differentiation of mESCs. These results suggest that, unlike *Lefty1*, the function of *Lefty2* in mESCs is more than a simple Nodal inhibitor. Indeed, inhibition of *Lefty* in *Xenopus* results in Nodal overexpression [21], indicating that the regulatory network of Nodal signaling and *Lefty* expression is more complicated than we understand.

Overexpression of *Lefty1* in the differentiating *Lefty2* KD explains that *Lefty2* functions to adjust the intensity of Nodal signaling at the crossroads of self-renewal and differentiation of mESCs. Even though enhanced expression of *Oct4* and *Sox2* is thought to be the main cause of *Lefty1* overexpression in the differentiating *Lefty2* KD cells, we cannot exclude the presence of additional mechanisms that coordinate the expression of *Lefty1* and *Lefty2* during the differentiation of mESCs. Further studies are necessary to uncover the mechanism by which *Lefty2* regulates the differentiation commitment of mESCs and regulates *Lefty1* expression. Regardless of Smad2 phosphorylation and the differentiation patterns of *Lefty1* KD and *Lefty2* KD cells, phosphorylation of Smad1 and 5, which are pivotal intracellular effectors of the bone morphogenetic protein (BMP), was suppressed in differentiating *Lefty1* KD and *Lefty2* KD cells (Supplementary Fig. S8). The intracellular TGF-β signaling network, which is finely tuned by ligands such as nodal and BMP (as well as by Lefty1/2 inhibitors) during the differentiation of mESCs, remains to be elucidated.

The study of *Lefty1* or *Lefty2* function in human ESCs also remains to be elucidated to understand the evolutionary conservation of Nodal signaling and Lefty activity.

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Author Disclosure Statement

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References

1. Seuntjens E, L Umans, A Zwijsen, M Sampaolesi, CM Verfaillie and D Huylebroeck. (2009). Transforming growth factor type beta and Smad family signaling in stem cell function. Cytokine Growth Factor Rev 20:449–458.

2. Adjaye J, J Huntriss, R Herwig, A BenKahla, TC Brink, C Wierling, C Hultschig, D Groth, ML Yaspo, et al. (2005). Primary differentiation in the human blastocyst: comparative molecular portraits of inner cell mass and trophectoderm cells. Stem Cells 23:1514–1525.

3. Richards M, SP Tan, JH Tan, WK Chan and A Bongso. (2004). The transcriptome profile of human embryonic stem cells as defined by SAGE. Stem Cells 22:51–64.

4. Sperger JM, X Chen, JS Draper, JE Antosiewicz, CH Chon, SB Jones, JD Brooks, PW Andrews, PO Brown and JA Thomson. (2003). Gene expression patterns in human embryonic stem cells and human pluripotent germ cell tumors. Proc Natl Acad Sci U S A 100:13350–13355.

5. Nakatake Y, N Fukui, Y Iwamatsu, S Masui, K Takahashi, R Yagi, K Yagi, J Miyazaki, R Matoba, MS Ko and H Niwa. (2006). Klf4 cooperates with Oct3/4 and Sox2 to activate the Lefty1 core promoter in embryonic stem cells. Mol Cell Biol 26:7772–7782.

6. Sekkai D, G Gruel, M Herry, V Moucadel, SN Constantinescu, O Albagli, D Tronik-Le Roux, W Vainchenker and A Bennaceur-Griscelli. (2005). Microarray analysis of LIF/Stat3 transcriptional targets in embryonic stem cells. Stem Cells 23:1634–1642.

7. Dvash T, Y Mayshar, H Darr, M McElhaney, D Barker, O Yanuka, KJ Kotkow, LL Rubin, N Benvenisty and R Eiges. (2004). Temporal gene expression during differentiation of human embryonic stem cells and embryoid bodies. Hum Reprod 19:2875–2883.

8. Oulad-Abdelghani M, C Chazaud, P Bouillet, MG Mattei, P Dolle and P Chambon. (1998). Stra3/lefty, a retinoic acid-inducible novel member of the transforming growth factor-beta superfamily. Int J Dev Biol 42:23–32.

9. Kosaki K, MT Bassi, R Kosaki, M Lewin, J Belmont, G Schauer and B Casey. (1999). Characterization and mutation analysis of human LEFTY A and LEFTY B, homologues of murine genes implicated in left-right axis development. Am J Hum Genet 64:712–721.

10. Yashiro K, Y Saijoh, R Sakuma, M Tada, N Tomita, K Amano, Y Matsuda, M Monden, S Okada and H Hamada. (2000). Distinct transcriptional regulation and phylogenetic divergence of human LEFTY genes. Genes Cells 5:343–357.

11. Schier AF and MM Shen. (2000). Nodal signalling in vertebrate development. Nature 403:385–389.

12. Cheng SK, F Olale, AH Brivanlou and AF Schier. (2004). Lefty blocks a subset of TGFbeta signals by antagonizing EGF-CFC coreceptors. PLoS Biol 2:E30.

13. James D, AJ Levine, D Besser and A Hemmati-Brivanlou. (2005). TGFbeta/activin/nodal signaling is necessary for the maintenance of pluripotency in human embryonic stem cells. Development 132:1273–1282.

14. Park KS, Y Cha, CH Kim, HJ Ahn, D Kim, S Ko, KH Kim, MY Chang, JH Ko, et al. (2013). Transcription elongation factor Tcea3 regulates the pluripotent differentiation potential of mouse embryonic stem cells via the Lefty1-Nodal-Smad2 pathway. Stem Cells 31:282–292.

15. Jirmanova L, M Afanassieff, S Gobert-Gosse, S Markossian and P Savatier. (2002). Differential contributions of ERK and PI3-kinase to the regulation of cyclin D1 expression and to the control of the G1/S transition in mouse embryonic stem cells. Oncogene 21:5515–5528.

16. Fei T, S Zhu, K Xia, J Zhang, Z Li, JD Han and YG Chen. (2010). Smad2 mediates Activin/Nodal signaling in mesendoderm differentiation of mouse embryonic stem cells. Cell Res 20:1306–1318.

17. Chan RJ, SA Johnson, Y Li, MC Yoder and GS Feng. (2003). A definitive role of Shp-2 tyrosine phosphatase in mediating embryonic stem cell differentiation and hematopoiesis. Blood 102:2074–2080.

18. Shiratori H, R Sakuma, M Watanabe, H Hashiguchi, K Mochida, Y Sakai, J Nishino, Y Saijoh, M Whitman and H Hamada. (2001). Two-step regulation of left-right asymmetric expression of Pitx2: initiation by nodal signaling and maintenance by Nkx2. Mol Cell 7:137–149.

19. Blum B, O Bar-Nur, T Golan-Lev and N Benvenisty. (2009). The anti-apoptotic gene survivin contributes to teratoma formation by human embryonic stem cells. Nat Biotechnol 27:281–287.

20. Cavallari C, V Fonsato, MB Herrera, S Bruno, C Tetta and G Camussi. (2013). Role of Lefty in the antitumor activity of human adult liver stem cells. Oncogene 32:819–826.

21. Branford WW and HJ Yost. (2002). Lefty-dependent inhibition of Nodal- and Wnt-responsive organizer gene expression is essential for normal gastrulation. Curr Biol 12: 2136–2141.

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