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Dissecting Signaling Pathways That Govern Self-renewal of Rabbit Embryonic Stem Cells*[S]

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The pluripotency and self-renewal of embryonic stem cells (ESC) are regulated by a variety of cytokines/growth factors with some species differences. We reported previously that rabbit ESC (rESC) are more similar to primate ESC than to mouse ESC. However, the signaling pathways that regulate rESC self-renewal had not been identified. Here we show that inhibition of the transforming growth factor β (TGFβ), fibroblast growth factor (FGF), and canonical Wnt/β-catenin (Wnt) pathways results in enhanced differentiation of rESC accompanied by down-regulation of Smad2/3 phosphorylation and β-catenin expression and up-regulation of phosphorylation of Smad1 and β-catenin. These results imply that the TGFβ, FGF, and Wnt pathways are required for rESC self-renewal. Inhibition of the MAPK/ERK and PI3K/AKT pathways, which lie downstream of the FGF pathway, led to differentiation of rESC accompanied by down-regulation of phosphorylation of ERK1/2 or AKT, respectively. Long-term self-renewal of rESC could be achieved by adding a mixture of TGFβ ligands (activin A, Nodal, or TGFβ1) plus basic FGF (bFGF) and Noggin in the absence of serum and feeder cells. Our findings also suggest that there is a regulatory network consisting of the FGF, Wnt, and TGFβ pathways that controls rESC pluripotency and self-renewal. We conclude that bFGF controls the stem cell properties of rESC both directly and indirectly through TGFβ or other pathways, whereas the effect of Wnt on rESC might be mediated by the TGFβ pathway.

Embryonic stem cells (ESC)[5] self-renew indefinitely and give rise to derivatives of all three primary germ layers and extraembryonic tissues. Because of their potential to provide a variety of tissues for use in regenerative medicine, there is great interest in studying the signaling pathways that regulate self-renewal of ESC. However, the signaling pathways that govern the unique properties of ESC remain largely unknown and exhibit some species differences.

Previous studies demonstrated that transforming growth factor β (TGFβ) (1), fibroblast growth factor (FGF) (2), and canonical Wnt/β-catenin (Wnt) (3) signaling pathways play prominent roles in the early embryogenesis of vertebrates. These pathways, together with the leukemia inhibitory factor (LIF)-signal transducers and activators of transcription 3 (Stat3) and bone morphogenetic protein (BMP) pathways (4–8), are also involved in the self-renewal of ESC, with some species differences. However, the precise functions and interactions of these pathways in regulating ESC are largely unknown. In mouse ESC (mESC), the LIF-Stat3 and BMP signaling pathways play essential roles in pluripotency maintenance (4–8). However, neither the addition of LIF to the culture medium (9, 10) nor the activation of Stat3 sustains the pluripotency of human and nonhuman primate ESC (11, 12). Furthermore, BMP promotes human ESC (hESC) to differentiate into trophoblasts (13), and the TGFβ pathway (another branch of the TGFβ superfamily, which contains two main branches, the BMP and TGFβ pathways), plays a key role in the maintenance of hESC pluripotency (14–18). The first indication of such a role emerged from the prevalence of TGFβ signaling pathway components in the transcriptome of hESC and their rapid disappearance upon differentiation (19). Moreover, exposing hESC to Nodal up-regulated pluripotent markers

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Table 1.

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[5] The abbreviations used are: ESC, embryonic stem cells; rESC, rabbit embryonic stem cells; mESC, mouse embryonic stem cells; hESC, human embryonic stem cells; TGFβ, transforming growth factor β; FGF, fibroblast growth factor; bFGF, basic fibroblast growth factor; Wnt, canonical Wnt/β-catenin; LIF, leukemia inhibitory factor; Stat3, signal transducers and activators of transcription 3; BMP, bone morphogenetic protein; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol-3 kinase; Akp, alkaline phosphatase; pSmad, phospho-Smad; pCatenin, phospho-β-catenin; CM, conditioned medium; EB, embryoid body; MEF, mouse embryonic fibroblast; FACS, fluorescence-activated cell sorting; SCID, severe combined immunodeficiency.

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(20); TGFβ family members are also less potent than other growth factors in inducing differentiation (21). However, the TGFβ pathway is only involved in the propagation of mESC without affecting their pluripotency (22). There is ample evidence of a role for basic FGF (bFGF) in hESC self-renewal; bFGF has been included in media to derive and maintain hESC lines, which suggests that the FGF pathway has an important function in regulating the pluripotency and self-renewal of hESC (21, 23–25). Mitogen-activated protein kinase (MAPK)/ERK and phosphatidylinositol-3 kinase (PI3K)/AKT pathways, both of which lie downstream of the FGF pathway, are important for maintenance of pluripotency and viability in hESC (2, 26–28). However, FGF stimulation of the ERK1/2 signaling cascade triggers the transition of mESC from self-renewal to lineage commitment (29), and self-renewal of mESC is enabled by the elimination of differentiation-inducing signaling from MAPK (30). On the other hand, inhibition of PI3K/AKT pathway leads to a reduction in the ability of LIF to maintain self-renewal in mouse ESC through augmenting LIF-induced phosphorylation of ERK (31). Wnt signaling was reported to be activated in undifferentiated human and mouse ESC (32), but Wnt activity alone is not sufficient to maintain pluripotency of hESC (16). The level of Wnt signaling is low in human ESC but increases during differentiation, and inhibiting Wnt signaling does not affect the self-renewal of hESC (33). Although signaling pathways that sustain monkey ESC self-renewal have not yet been well defined, it appears that exogenous LIF or bFGF is absolutely required for rhesus (10, 34) or marmoset (35) monkey ESC. These previous studies suggested that the LIF-Stat3, TGFβ, FGF, and Wnt pathways could be potential candidates for regulating ESC pluripotency and self-renewal but that there exist some species differences in the mechanisms regulating pluripotency maintenance of ESC.

In our previous study, pluripotent rabbit ESC (rESC) lines were established from fertilized and parthenogenetic embryos. The rESC expressed all cell surface markers found in both mouse and primate ESC. rESC were more similar to primate ESC than mouse ESC in many ways, including morphological characteristics, LIF-independent self-renewal, and trophoblast differentiation capacity (36). However, the signaling pathways involved in the pluripotency and self-renewal of rESC are still largely unexplored. Here, we tackled this question by using activators and inhibitors of various signaling pathways and found that activation of the TGFβ, FGF (MAPK/ERK and PI3K/AKT), and Wnt pathways and inhibition of the BMP pathway are required for rESC self-renewal. Mixed media composed of ligands of TGFβ (TGFβ1, activin A, or Nodal) and bFGF plus a BMP antagonist were sufficient to sustain long-term culture of the rESC lines. Our findings also suggest that there is an interactive network among the FGF, Wnt, and TGFβ pathways that regulates rESC self-renewal.

EXPERIMENTAL PROCEDURES

Culture of rESC—Two rESC lines, RF and RP01, derived from a fertilized and a parthenogenic blastocyst, respectively, were cultured as described previously (36). Briefly, the cells were cultured on mouse embryonic fibroblast (MEF) in ESC medium containing Dulbecco's modified Eagle's medium (Invitrogen Corp.) supplemented with 10% defined fetal bovine serum (Hyclone, Logan, UT), 2 mM L-glutamine (Sigma-Aldrich), 1% non-essential amino acids (Invitrogen), and 0.1 mM β-mercaptoethanol (Sigma-Aldrich). The rESC from passages 30 to 40 (p30–p40) were used in this study.

For receptor inhibition experiments, rESC were treated with 1 mg/ml dispase (Invitrogen) for 10 min to detach them from the feeder cells. The dislodged cell colonies were then dissociated into single cells by treatment with 0.25% trypsin (Sigma-Aldrich) in 0.04% EDTA (Sigma-Aldrich) solution (23). The cells (10^3/cm^2) were inoculated onto new feeders in ESC medium or onto Matrigel (Sigma-Aldrich) in MEF-conditioned ESC medium (MEF-CM) as described for the culture of hESC (37). The rESC were then treated with or without various inhibitors or combinations of inhibitors including: SB431542 (Tocris Bioscience, Northpoint, UK), an inhibitor of type I receptors (ALK4, ALK5, and ALK7) of the TGFβ/activin/Nodal-initiated Smad2/3 pathway (16, 38, 39); TGFβ receptor I (ALK5) inhibitor (Calbiochem), an inhibitor of the TGFβ-initiated Smad2/3 pathway (40, 41); SU5402 (Calbiochem), an inhibitor of the tyrosine kinase of FGF receptor I (42); Frizzled-1 (R&D Systems, Minneapolis, MN), an antagonist of the Wnt pathway (43); and anti-Wnt3a antibody (R&D Systems), also an inhibitor of the Wnt pathway via neutralization of Wnt3a bioactivity; PD98059 (Promega Corp., Madison WI), an inhibitor of MAPK/ERK pathway; LY294002 (Cell Signaling Technology, Danvers, MA), an inhibitor of PI3K/AKT pathway (26–31).

For feeder- and serum-free cultures, rESC colonies were detached from the feeder cells after treatment with 1 mg/ml dispase for 10 min and then broken into small cell clumps by gentle pipetting. The cell clumps were seeded onto Matrigel (Sigma-Aldrich)-coated plates (Nunc A/S, Roskilde, Denmark) in KSR medium containing knock-out Dulbecco's modified Eagle's medium, 20% knock-out serum replacement, 0.1 mM β-mercaptoethanol, 1% nonessential amino acids, and 2 mM glutamine supplemented with activin A, Nodal, TGFβ1, BMP4, Wnt3a (all from R&D Systems), bFGF (Chemicon International, Temecula, CA), Noggin (Sigma-Aldrich), or various combinations of the above factors. The cells were split every 3–4 days.

The differentiation potential of rESC from feeder- and serum-free culture systems was tested by *in vitro* embryoid body (EB) formation and *in vivo* teratoma formation assays. Dislodged rESC colonies were suspended in the ESC medium and cultured in hanging drops (30 μl/drop). Two days later, the formed EBs were transferred to Petri dishes (BD Biosciences) coated with agar (Sigma-Aldrich) to keep them in a continuous suspension culture for another 2 days. Then the EBs were plated onto gelatin (Sigma-Aldrich)-coated plates and continuously cultured in the ESC medium until being harvested for analysis of the differentiation markers. For teratoma formation, 6–8-week-old Severe Combined Immunodeficiency (SCID)-beige mice (Charles River Laboratory) were injected intramuscularly with $2–4 \times 10^6$ rESC. The tumors were removed 8–14 weeks after injection and fixed in 4% paraformaldehyde. Paraffin sections were stained with hematoxylin and eosin and processed for histological examination.

Characterization of ESC—In situ analysis of pluripotency markers such as alkaline phosphatase (Akp; Sino-American

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Biotechnology Co., LuoYang, China), SSEA-4 (Chemicon International), and Oct4 (Santa Cruz Biotechnology, Santa Cruz, CA) on rESC or their derivatives was performed as described previously (36). Based on Akp staining, rESC colonies were classified into two types: undifferentiated (compacted colonies with almost all cells positive for Akp) and differentiated (non-compacted colonies with some or all cells negative for Akp) (44). The percentage of differentiated cell colonies compared with the total number of colonies was determined. Antibodies used to immunostain other markers included those against Nestin and glial fibrillary acidic protein (Chemicon International), brachyury (Santa Cruz Biotechnology), phospho-Smad1/5/8 (pSmad1/5/8) and phospho-Smad2/3 (pSmad2/3) (both from Cell Signaling Technologies), α-fetoprotein and albumin (Sigma-Aldrich), and smooth muscle actin (DakoCytomation, Glostrup, Denmark). The staining was visualized using fluorescein isothiocyanate- or phycoerythrin-conjugated secondary antibody (Santa Cruz Biotechnology). Cell nuclei were counterstained with Hoechst 33342 (Sigma-Aldrich). Images were taken using a confocal laser-scanning system LSM 510 META (LSM 510 META; Carl Zeiss, Jena, Germany).

Cells positive for SSEA-4 or Oct4 were quantified by fluorescence-activated cell sorting (FACS). The cells were detached from the plates with 1 mg/ml dispase and dissociated with 0.25% trypsin in 0.04% EDTA (Sigma-Aldrich) (23). Cells to be probed for the cell surface marker SSEA-4 were tested live, whereas cells to be probed for the nuclear marker Oct4 were fixed with 0.1% paraformaldehyde (Sigma-Aldrich) for 10 min at 37 °C and then permeabilized with 90% methanol (Fisher Scientific) for 30 min on ice. 10^5 fixed (for Oct4) or live (for SSEA-4) cells were then incubated for 30 min at room temperature with anti-SSEA-4 antibody at a 1:50 dilution or with anti-Oct4 antibody at a 1:100 dilution. The corresponding isogenic immunoglobins (Santa Cruz Biotechnology) were used as negative controls for gating. The cells were washed twice in phosphate-buffered saline containing 3% normal goat serum (Sigma-Aldrich) and then incubated with fluorescein isothiocyanate-conjugated secondary antibody (Santa Cruz Biotechnology) at a 1:200 dilution. The percentage of $Oct4^+$ or $SSEA\text{-}4^+$ cells was calculated following FACS analysis. Cell expansion -fold after a treatment was calculated as the number of $SSEA\text{-}4^+$ cells at the end of the treatment over the total number of cells plated before the treatment. Two or more replicates were set for each group, and all experiments were repeated multiple times. The karyotypes of rESC were analyzed as described by Hayes *et al*. (45).

Western Blotting—Cell colonies were mechanically removed from the culture plates and washed with phosphate-buffered saline. Each cell sample was divided into two groups, from which the nuclear and total proteins were extracted, respectively. For nuclear protein extraction, the cell pellet was resuspended in a lysis buffer containing 10 mM Hepes, 10 mM KCl, 0.1 mM EDTA, and 0.1 mM EGTA with proteinase inhibitors to which 0.5 mM phenylmethylsulfonyl fluoride was added just before use. After a 15-min incubation at 4 °C, 1% Nonidet P-40 was added, and following centrifugation (11,400 × *g* for 5 min) the nuclei were precipitated and lysed in a buffer containing 10 mM Hepes, 0.4 mM NaCl, and 5 mM EDTA. After incubation for 30 min at 4 °C and centrifugation at 11,400 × *g* for 5 min, the supernatant containing the nuclear extract was transferred into a fresh tube and the protein content estimated as described below.

For total protein extraction, radioimmune precipitation assay buffer, which contained 65 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, and 0.25% sodium deoxycholate, was used to lyse the cells and was supplemented with proteinase inhibitors before use. The cell pellets were resuspended and incubated in the radioimmune precipitation assay buffer for 30 min at 4 °C. The cell lysates were centrifuged at 12,000 × *g* for 8 min at 4 °C. Quantification of protein concentrations was carried out using the Protein Quantification Kit-Rapid (Biochemica International, Melbourne, FL) according to the manufacturer's instructions.

The protein extracts (20 μg/lane) were separated in a polyacrylamide gel and transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA). The membrane was then stained with primary antibodies to detect pSmad1/5/8, pSmad2/3, β-catenin (Abcam International), or Oct4 in the nuclear extracts, with AKT, phospho-AKT (Ser-473, p-AKT), phospho-ERK1/2 (Thr-202/Tyr-204, p-ERK1/2), ERK1/2 (all from Cell Signaling Technologies), Smad4 (Abcam), phospho-β-catenin (pCatenin, Abcam), bFGF (Calbiochem), or glyceraldehyde-3-phosphate dehydrogenase (as a loading control, Kangchen Bio-Tech, Shanghai, China) for the total extracts. Horseradish peroxidase-labeled corresponding secondary antibodies (Pierce Biotechnology) were used to bind the primary antibodies. The antibody-bound protein bands on the membranes were visualized by using the ECL Western blotting detection system (Pierce). The experiments were repeated at least once on three different batches of rESC.

Statistical Analysis—The results are presented as means ± S.E. Statistical analysis was performed using the least significant difference test. Statistical significance was defined as $p < 0.05$.

RESULTS

TGFβ, FGF, and Wnt Pathways Are Required for rESC Self-renewal—The rESC express many genes for components of the TGFβ, FGF, and Wnt signaling pathways (36), but whether these pathways are required to sustain the self-renewal of rESC remains elusive. In this study, we first addressed this question by using SB431542 and TGFβ receptor I inhibitor (to repress the TGFβ pathway), SU5402 (to repress the FGF pathway), and Frizzled-1 and anti-Wnt3a antibody (to repress the Wnt pathway). These inhibitors promoted differentiation of rESC either on MEF in the ESC medium or on Matrigel in MEF-CM as judged on the basis of morphological criteria, Akp activity, and Oct4 expression (Table 1 and Fig. 1). Inhibition of the TGFβ, FGF, or Wnt pathways had negative effects on the pluripotency and proliferation capacity of rESC, based on up-regulation of the cell differentiation rate, the fraction of SSEA-4 positive cells, and the cell expansion -fold (Table 1). The results also showed that the degree of inhibition was different for different inhibitors. The efficacy of these inhibitors was in the following high-to-low order: SB431542 or SU5402 > TGFβ receptor I inhibitor > Frizzled-1 or anti-Wnt3a antibody

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TABLE 1

Inhibitors of the FGF, TGFβ, and Wnt pathways suppress rESC self-renewal

Inhibitors of the FGF, TGFβ, and Wnt pathways suppress rESC self-renewal. Dissociated RF line rESC were cultured on MEFs in ESC medium (ESM) or on Matrigel in MEF-CM for 5 days with or without 10 μM SU5402 (SU), SB431542 (SB), TGFβ receptor I inhibitor (TI), 200 ng/ml Frizzled-1 (Fz), 20 μg/ml anti-Wnt3a antibody, or various combinations of the inhibitors or their vehicle control, 0.1% DMSO. Percentages of differentiated cell colonies (by *in situ* Akp staining), percentages of $SSEA4^+$ cells, and cell expansion -fold (by FACS) were determined (see "Experimental Procedures"). All treatments were performed in triplicate, and the experiment was repeated multiple times. Data are displayed as means ± S.E. The least significant difference test was used for statistical analysis. Groups marked with different alphabetical superscripts (*a–f*) in the columns are significantly different from each other ($p < 0.05$). Similar results were obtained with the parthenogenic rESC line RP01 (not shown).

Treatment	Fraction of differentiated cell colonies	Fraction of SSEA-4 expression	Cell expansion
	%	%	-fold
RF cultured on MEFs in ESM			
Control (ESM)	4.45 ± 0.21[a]	88.14 ± 2.31[a]	12.33 ± 1.17[a]
ESM + SU	72.18 ± 0.97[b]	44.72 ± 1.08[b]	5.83 ± 0.44[b]
ESM + SB	75.53 ± 2.20[b]	41.57 ± 1.67[b]	5.17 ± 0.44[b]
ESM + TI	60.27 ± 1.07[c]	60.25 ± 1.17[c]	8.17 ± 1.07[c]
ESM + Fz	38.34 ± 2.18[d]	72.20 ± 1.03[d]	9.67 ± 1.45[d]
ESM + anti-Wnt3a	32.08 ± 1.94[d]	69.62 ± 0.61[d]	9.78 ± 1.03[d]
ESM + SU + Fz	84.32 ± 1.22[e]	32.54 ± 1.56[e]	5.01 ± 0.78[b]
ESM + SB + Fz	73.46 ± 1.09[b]	39.69 ± 0.80[b]	5.20 ± 0.65[b]
ESM + SU + SB	96.52 ± 0.99[f]	14.45 ± 0.59[f]	2.01 ± 0.48[e]
ESM + 0.1% DMSO	4.40 ± 0.23[a]	86.66 ± 1.62[a]	12.50 ± 0.88[a]
RF cultured in MEF-CM without feeders			
Control (ES)	5.97 ± 0.97[a]	87.12 ± 1.71[a]	12.83 ± 0.60[a]
ESM + SU	80.23 ± 0.87[b]	43.75 ± 1.34[b]	5.83 ± 0.17[b]
ESM + SB	81.60 ± 1.19[b]	42.15 ± 0.46[b]	5.67 ± 0.44[b]
ESM + TI	65.76 ± 1.43[c]	56.94 ± 1.32[c]	7.83 ± 0.17[c]
ESM + Fz	45.33 ± 1.75[d]	69.77 ± 0.78[d]	8.96 ± 0.58[d]
ESM + anti-Wnt3a	41.68 ± 0.94[d]	68.26 ± 1.27[d]	9.04 ± 1.01[d]
ESM + SU + Fz	89.38 ± 0.96[e]	32.10 ± 0.89[e]	4.89 ± 0.39[c]
ESM + SB + Fz	80.87 ± 1.29[b]	40.60 ± 1.37[b]	5.41 ± 0.76[c]
ESM + SU + SB	98.46 ± 1.42[f]	14.34 ± 0.68[f]	2.26 ± 0.84[b]
ESM + 0.1% DMSO	5.25 ± 1.08[a]	87.57 ± 1.27[a]	12.67 ± 0.73[a]

(Table 1 and Fig. 1). A combination of the inhibitors showed a synergistic effect between SB431542 and SU5402. Furthermore, the combination of SU5402 and Frizzled-1 increased the inhibition efficacy compared with SU5402 addition alone. However, the addition of Frizzled-1 did not reinforce or rescue the inhibitory effects of SB431542 (Table 1). Similar data were obtained with the parthenogenic rESC line, RP01 (data not shown). These results suggest that the TGFβ, FGF, and Wnt pathways are all required for rESC self-renewal and proliferation, with the TGFβ and FGF pathways playing a major role.

Of the two arms of TGFβ superfamily signaling, the TGFβ/activin/Nodal-initiated SMAD2/3 pathway is activated and the BMP-initiated SMAD1/5/8 pathway is inhibited in human ESC (15, 16, 18, 46–49). We tested the status of these two arms in the rESC lines RF and RP01 cultured on MEFs. We found that Smad2/3 were phosphorylated and localized to the nuclei of undifferentiated rESC but that this process was inhibited in rESC treated with the inhibitors of the TGFβ or FGF pathway (Fig. 1, *A* and *B*). On the other hand, Smad1/5/8 phosphorylation was barely evident in undifferentiated rESC but increased and was localized to the nucleus in rESC treated with the inhibitors (Fig. 1, *A* and *B*). Inhibition of the Wnt pathway also reduced the level of pSmad2/3 as well as that of β-catenin, whereas it enhanced pSmad1/5/8 and pCatenin (Fig. 1, *A* and *C*). On the other hand, inhibition of the TGFβ and FGF pathways reduced the levels of β-catenin and bFGF (Fig. 1*B*). There was no change in the expression of Smad4 during the differentiation stage (Fig. 1*B*). These results suggest that TGFβ and Wnt signaling are activated and BMP signaling is inhibited in rESC, which are maintained by the TGFβ, FGF, and Wnt pathways together.

MAPK/ERK and PI3K/AKT Pathways Are Required for Maintaining rESC in an Undifferentiated State—MAPK/ERK and PI3K/AKT pathways lie downstream of the FGF pathway and regulate survival, proliferation, apoptosis, and fate determination in a diverse set of cells (2). Our results show that the FGF pathway is important in maintaining pluripotency and viability in rESC (Table 1 and Fig. 1), but whether these two cascades play key role in the maintenance of pluripotency and self-renewal in rESC need to be further studied. Here, we treated rESC cultured in the presence of CM with different concentration of PD98059 (to repress the MAPK/ERK pathway) and LY294002 (to repress the PI3K/AKT pathway). Both of these inhibitors promoted differentiation of rESC with decreased expression of the pluripotency markers Oct4 and SSEA-4, and differentiation was also enhanced when the concentrations of PD98059 and LY294002 were further elevated up to 40 μM (Fig. 2). Therefore, MAPK/ERK and PI3K/AKT signaling activity is suggested to be necessary for maintaining rESC in an undifferentiated state.

We analyzed the activation status of the MAPK/ERK and PI3K/AKT pathways. ERK1/2 and AKT were highly phosphorylated in undifferentiated rESC, and ERK1/2 and AKT phosphorylation was down-regulated in differentiated rESC cultured in KSR medium only (as a negative control), which indicated that these two cascades are activated in undifferentiated rESC (Fig. 2*C*). Inhibition of MAPK/ERK and PI3K/AKT pathways resulted in down-regulation of ERK1/2 and AKT phosphorylation, respectively. However, PD98059 or LY294002 had no any inhibitory effect on the phosphorylation of AKT or ERK1/2, respectively, suggesting that there is no cross-talk between these two pathways in rESC (Fig. 2).

The MAPK/ERK and PI3K/AKT signaling pathways have been shown to be downstream targets of the insulin-like growth factor, epidermal growth factor, and platelet derived-growth factor pathways, in addition to the FGF pathway, in most cell types. To demonstrate whether the FGF pathway contributes to the activation of MAPK/ERK and PI3K/AKT pathways, we analyzed the phosphorylation change of ERK1/2 and AKT in rESC when 10 or 100 ng/ml bFGF was added to the KSR culture system (as a negative control). The addition of bFGF resulted in a high level of ERK1/2 and AKT phosphorylation, close to that in the positive control supported by the MEF-CM; the levels of ERK1/2 and AKT phosphorylation in rESC cultured in KSR medium only were relatively weak compared with the positive control (Fig. 2*C*). These results suggest that both the MAPK/ERK and PI3K/AKT cascades are downstream targets of the FGF pathway in rESC.

Activation of the TGFβ and FGF Pathways and Inhibition of the BMP Pathway Promote rESC Self-renewal—We next determined what external factors are required and sufficient to maintain rESC self-renewal. RF and RP01 rESCs were seeded onto Matrigel-coated plates in the feeder- and serum-free KSR

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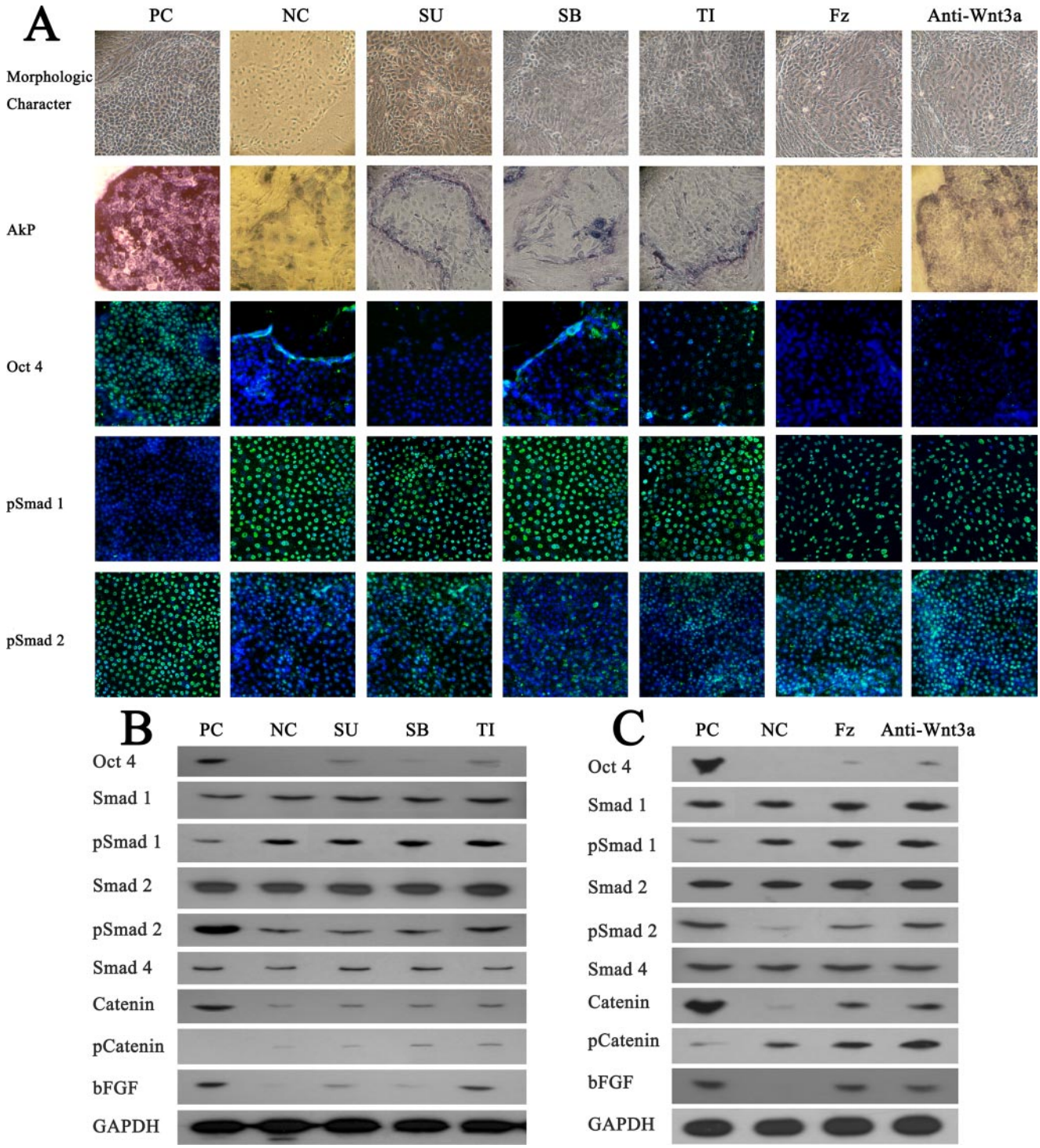

FIGURE 1. **Detection of markers in rESC treated with inhibitors of the TGFβ, FGF and Wnt pathways.** *In situ* detection (*A*) or Western blotting assays (*B* and *C*) were performed for markers in RF line rESC treated with or without various inhibitors for 5 days. rESC were cultured in the standard ESC medium with MEF as positive control (*PC*) or without MEF as negative control (*NC*). Inhibition of TGFβ, FGF, or Wnt signaling precipitated rabbit ESC differentiation (by morphology, Akp activity, and Oct4 expression analysis) accompanied by inactivation of Smad2/3 and activation of Smad1/5/8. The cell nuclei were counterstained with Hoechst 33342 (*blue*). *SB*, SB431542 (10 μM); *SU*, SU5402 (10 μM); *TI*, TGFβ receptor I inhibitor (10 μM); *Fz*, Frizzled-1/Fragment crystallizable chimera protein (200 ng/ml); *Anti-Wnt3a*, anti-Wnt3a antibody (20 μg/ml); *catenin*, β-catenin. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was tested in the Western blotting as an internal control. RF ESC was used in these representative micrographs, and similar results were obtained from RP01 ESC.

medium supplemented with the ligands for the TGFβ, FGF, and Wnt pathways, *e.g.* 1 ng/ml TGFβ1, 10 ng/ml activin A, 100 ng/ml Nodal, 10 or 100 ng/ml bFGF, 10 ng/ml BMP4, or 100 ng/ml Wnt3a, or their combinations (where 10 ng/ml bFGF was used). Noggin, an antagonist of BMP ligands, was added at 100 ng/ml to some combinations. The self-renewal-promoting effect of these factors was determined by FACS to quantify $Oct4^+$ and $SSEA\text{-}4^+$ cells cultured for 4 days at passage 1 on Matrigel. In the absence of these factors (KSR only as a negative control), most rESC differentiated as indicated by the very low

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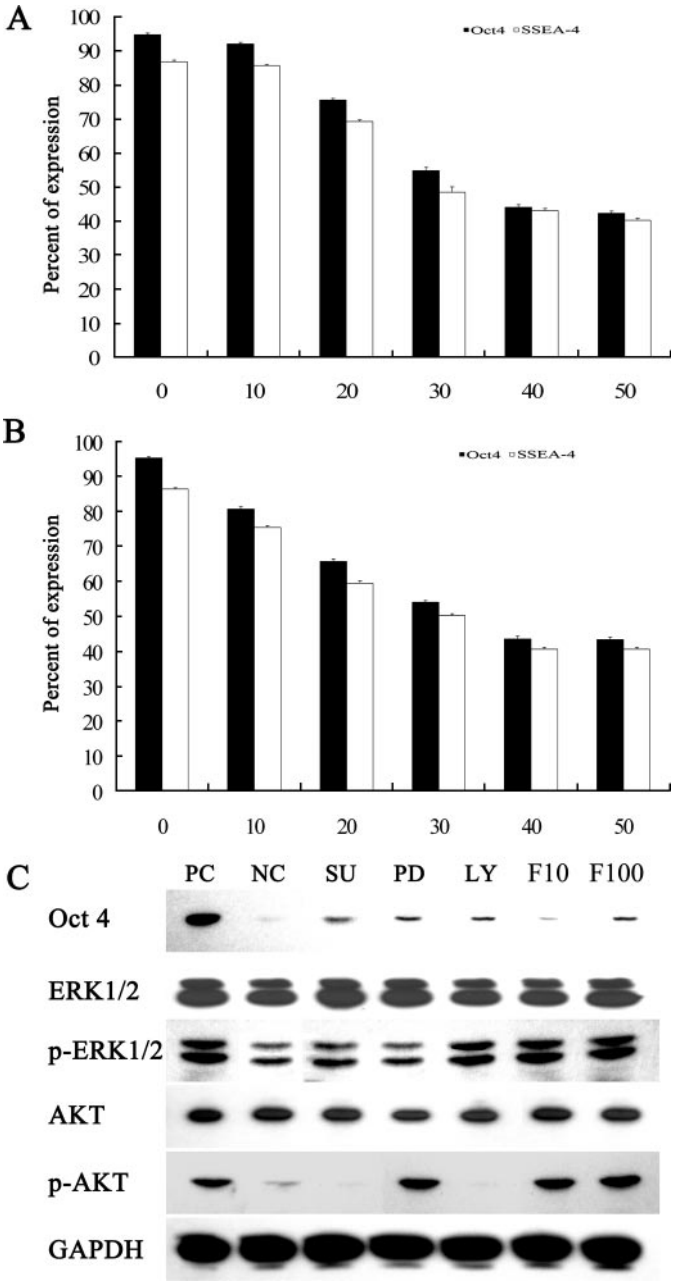

FIGURE 2. **The effect of the inhibitors of MAPK/ERK and PI3K/AKT pathway on the pluripotency maintenance of rESC.** FACS analysis for pluripotency markers Oct4 and SSEA-4 in RF line rESC treated with different concentration (from 0~50 μM) PD98059 (*A*) and LY294002 (*B*) for 5 days. All treatments were performed in triplicate, and the experiment was repeated multiple times. Statistical analysis was performed using the least significant difference test. *C*, Western blotting assay was performed for markers in RF line rESC treated with or without inhibitors or bFGF for 5 days. rESC were cultured in the MEF-CM as positive control (*PC*) or in KSR only as negative control (*NC*). *SU*, SU5402 (10 μM); *PD*, PD98059 (40 μM); *LY*, LY294002 (40 μM); *F10*, KSR+bFGF (10 ng/ml); *F100*, KSR+bFGF (100 ng/ml). Similar results were obtained from RP01 line rESC (not shown) *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

ratios of Oct4$^+$ and SSEA-4$^+$ cells. TGFβ1, activin A, Nodal, or bFGF (10 ng/ml or 100 ng/ml) alone enhanced the ratios of Oct4$^+$ and SSEA-4$^+$ cells. Although Wnt signaling is also active in rESC, Wnt3a did not promote rESC self-renewal (Fig. 3 and supplemental Table 1). These results suggest that ligands stimulating the TGFβ and FGF, but not the Wnt, pathways support rESC self-renewal but that none of them alone can fully prevent rESC differentiation.

Fig. 3 and supplemental Table 1 also show that SB431542 added to the KSR medium inhibited rESC self-renewal even in the presence of bFGF, whereas SU5402 had little effect on rESC self-renewal enhanced by activin A. The combination of bFGF with TGFβ1, activin A, or Nodal resulted in high ratios of Oct4$^+$ and SSEA-4$^+$ cells, close to that in the positive control supported by the MEF-CM. The addition of Noggin to the KSR medium supplemented with the above combined growth factors further increased the ratios of Oct4$^+$ and SSEA-4$^+$ cells, whereas the addition of BMP4 to the mixtures decreased the ratios. These data suggest that bFGF and any of the three TGFβ ligands are all beneficial to rESC self-renewal and that their combination can sustain high ratios of Oct4$^+$ and SSEA-4$^+$ cells comparable to that sustained by MEF-CM.

Combinations of Self-renewal-promoting Factors Support Long-term rESC Culture and Developmental Potential—Any culture formula for supporting ESC self-renewal has to be tested for a prolonged period to validate its effect convincingly. We tested the above mixtures on rESC of both the RF and RP01 lines cultured on Matrigel through sequential propagations. The combination of TGFβ ligands and bFGF sustained the supported rESC to maintain pluripotency markers, including Akp, SSEA-4, and Oct4, at the beginning of the first passage (Fig. 4*A*). At later passages, the cells gradually differentiated, accompanied by the loss of the pluripotency markers (Fig. 4*A*). However, the addition of Noggin to any of the combinations prevented subsequent differentiation and sustained the pluripotency markers and long-term self-renewal of both rESC lines, accompanied by activation of Smad2/3, inactivation of Smad1/5/8, up-regulation of β-catenin and bFGF, and down-regulation of pCatenin (Fig. 4, *A* and *B*). After 15 passages in these mixed media, rESC retained normal karyotypes (Fig. 4, *C* and *D*) and demonstrated the potential to differentiate into various cell types from the three germ layers in the EB and teratoma assays (Fig. 5). α-Fetoprotein and brachyury immunopositive cells were detected in the differentiated cell cultures after the EBs were continuously cultured for another 10–20 days, and albumin and muscle actin immunopositive cells were observed after 20 days. Small elongated cells (neural progenitor-like cells) were selected for culture, and most of these cells were Nestin-immunopositive; glial fibrillary acidic protein, as an astrocyte marker, was detected in the continuous culture (Fig. 5*A*). The rESC were injected intramuscularly into the rear legs of SCID-beige mice for 8–14 weeks. Teratoma recovery and analysis included representatives of all three germ layers: neural rosettes (ectoderm), bone (mesoderm), and endoderm epithelia (Fig. 5*B*).

DISCUSSION

Following our recent derivation of the four rESC lines (36), the present study was aimed at characterizing the signaling pathways that regulate self-renewal of rESC and the interactions among these pathways. Mouse ESC rely on both the LIF and BMP pathways to sustain their self-renewal (4–8), whereas human ESC self-renewal requires activation of both the FGF

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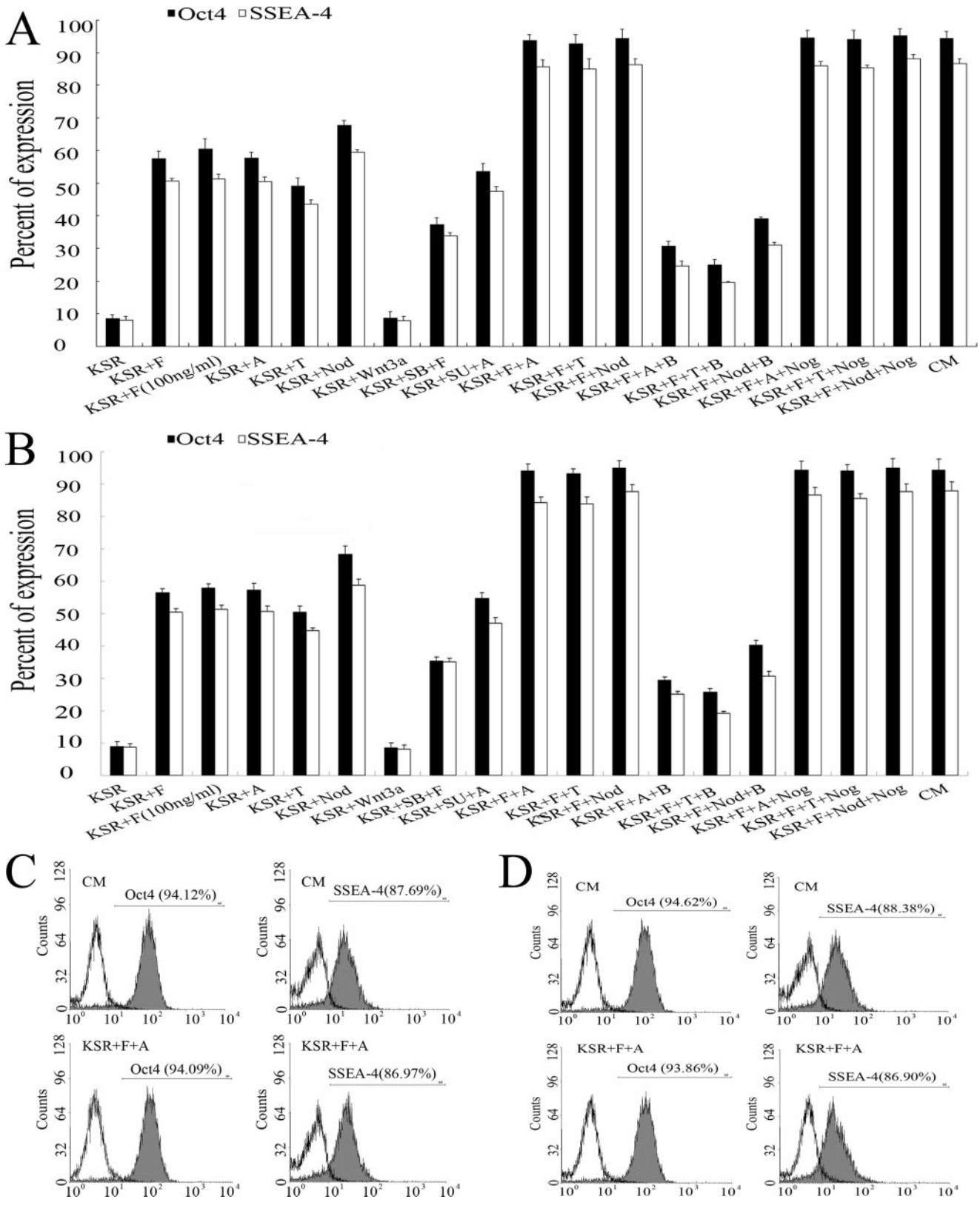

FIGURE 3. **FACS analysis for pluripotency markers in rESC of the RF (*A* and *C*) and RP01 (*B* and *D*) lines treated with or without exogenous ligands for the TGFβ, FGF, and BMP pathways and Noggin, SU5402, and SB431542.** Data are displayed as percentages (*A* and *B*) or histograms (*C* and *D*) of Oct4$^+$ and SSEA-4$^+$ rESC as representative of CM and KSR+F+A. The rESC were cultured on Matrigel in MEF-CM (CM, as a positive control) or unconditioned (KSR, as a negative control) for 4 days before FACS analysis. The ligands with or without Noggin were added to the KSR medium during the treatments. All treatments were performed in triplicate, and the experiment was repeated multiple times. Statistical analysis was performed using the least significant difference test. *KSR*, knock-out Dulbecco's modified Eagle's medium + 20% knock-out serum replacement + the designated supplements (see "Experimental Procedures"); *F*, bFGF (10 ng/ml); *A*, activin A (10 ng/ml); *T*, TGFβ1 (1 ng/ml); *Nod*, Nodal (100 ng/ml); *B*, BMP4 (10 ng/ml); *Nog*, Noggin (100 ng/ml); *Wnt3a*, recombinant mouse Wnt3a (100 ng/ml); *SU*, SU5402 (10 μM); *SB*, SB431542 (10 μM).

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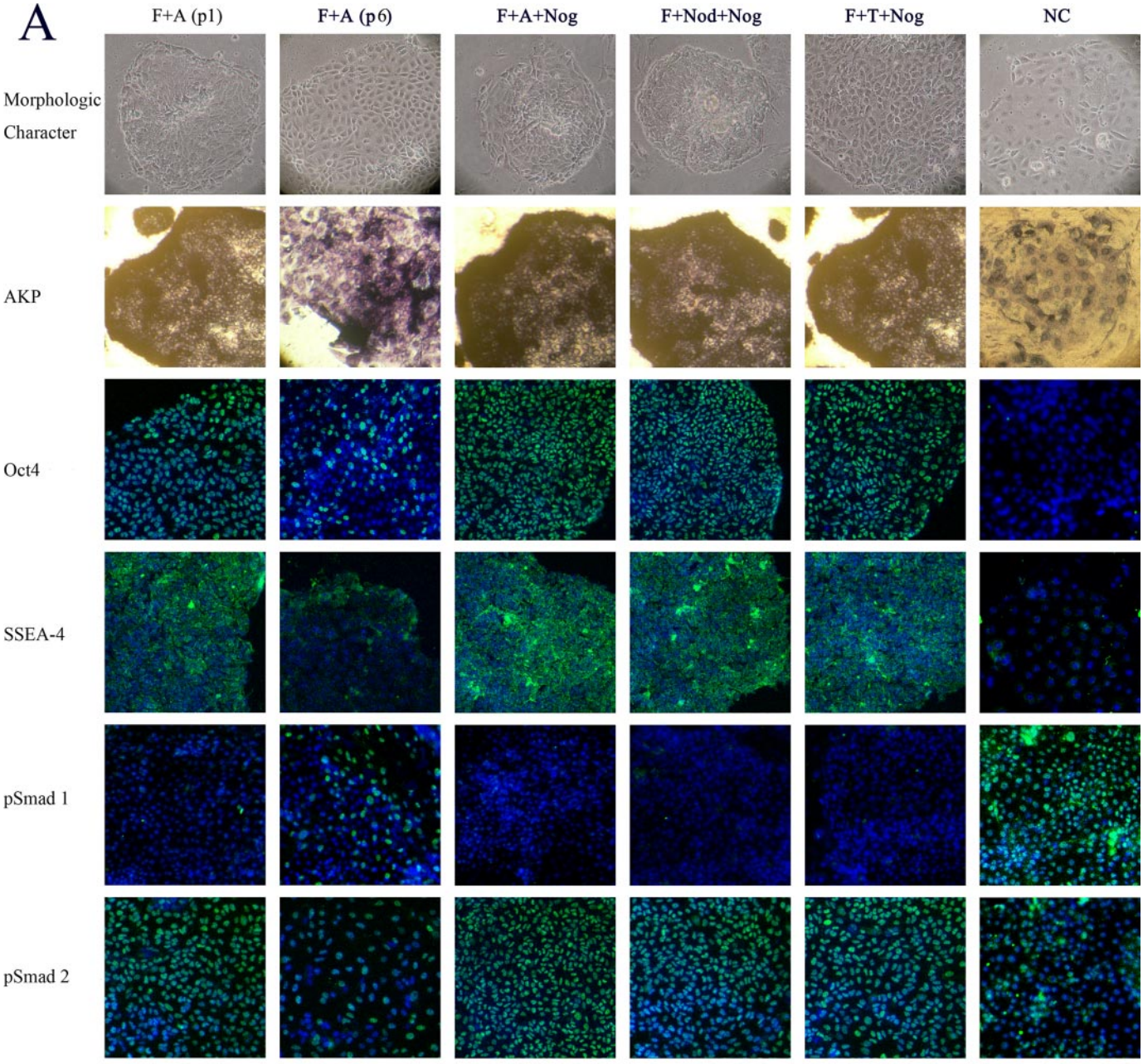
and TGFβ pathways and inhibition of the BMP pathway (18, 47, 50). The TGFβ pathway is only required for mouse ESC proliferation (22). These different requirements for various self-renewal factors have raised an obvious question: What signals regulate rESC self-renewal? Interestingly, rESC are more similar to human ESC, also requiring activated TGFβ and FGF sig-



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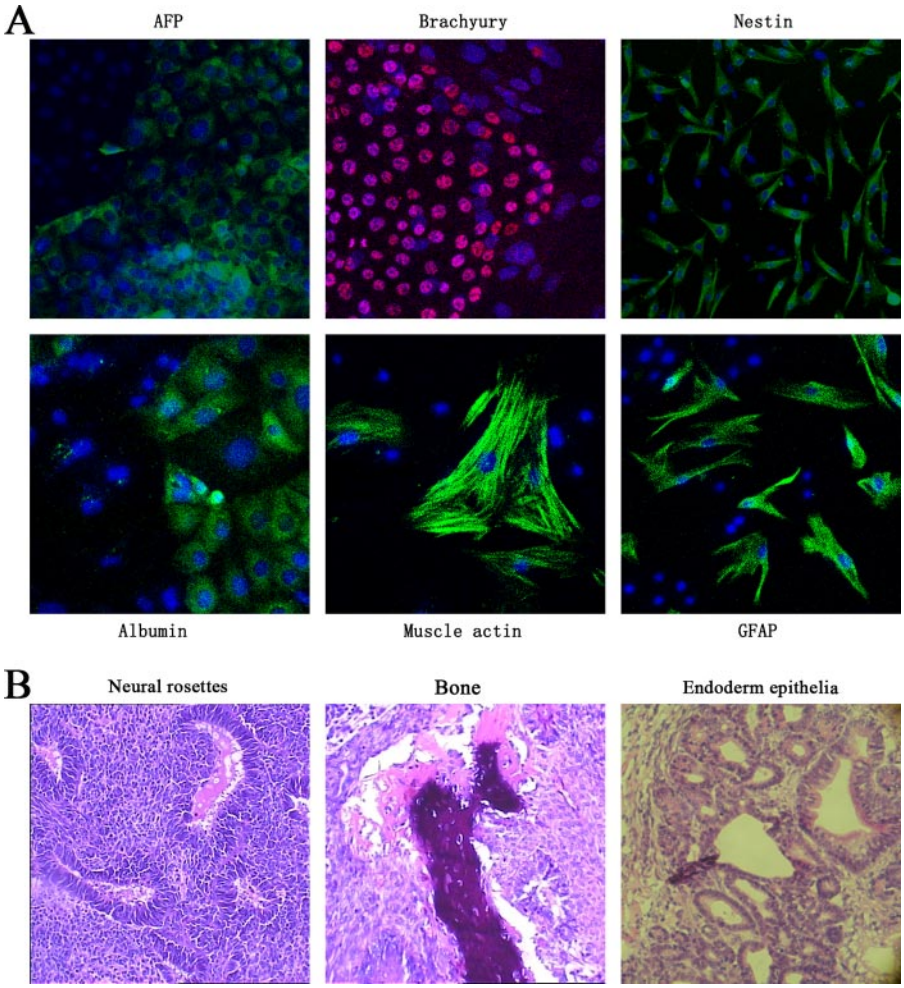

FIGURE 5. **Development potential of rESC supported by bFGF, activin A, and Noggin.** *A*, RF line rESC cultured on Matrigel in KSR medium supplemented with 10 ng/ml bFGF, 10 ng/ml activin A, and 100 ng/ml Noggin for 15 passages were used to form EBs. The EB cells were replated for further differentiation (see "Experimental Procedures") prior to immunostaining for markers of various cell types from the three germ layers. The cell nuclei were counterstained with Hoechst 33342 (*blue*). *AFP*, α-fetoprotein; *GFAP*, glial fibrillary acidic protein. *B*, rESC cultured as described above at passage 15 were injected into SCID-beige mice to form teratomas. Sections of the teratomas were subjected to hematoxylin and eosin staining. Representative tissues from the three germ layers are displayed. Similar results were obtained from the RP01 line rESC.

naling and inhibited BMP signaling, which implies that rESC will be a good model before the clinical application of human ESC in regenerative medicine. Both TGFβ and FGF signaling appear to be crucial for pluripotency maintenance and self-renewal of rESC.

These conclusions are supported by evidence from two sets of experiments through loss of function and gain of function, respectively. Inhibition of TGFβ-Smad2/3 signaling with SB431542 and inhibition of FGF signaling with SU5402 promoted rESC differentiation. Both scenarios were accompanied by inhibition of Smad2/3 phosphorylation and activation of Smad1/5/8 phosphorylation (Figs. 1 and 2). On the other hand, a combination of TGFβ1, activin A, or Nodal with bFGF and Noggin maintained long-term culture of rESC in a feeder- and serum-free culture system. Noggin helped the TGFβ ligands and bFGF to sustain rESC self-renewal. Without Noggin, these factors could only support rESC culture for several passages, and the cells eventually differentiated (Fig. 4). In contrast, exogenous TGFβ1 has little effect on human ESC self-renewal either in the KSR medium (18) or the defined TeSR medium (17). However, inhibition of TGFβ by the TGFβ receptor I inhibitor increased the differentiation frequency in rESC (Table 1, Fig. 1), and TGFβ1 reduced the differentiation of rESC when added alone and sustained rESC pluripotency for prolonged periods when combined with other growth factors (Figs. 3 and 4). These results indicated that, in addition to activin A and Nodal, TGFβ1 also plays a role in rESC pluripotency maintenance.

Another difference between rabbit and human ESC is reflected by the different roles of Wnt signaling in regulating their self-renewal. The level of Wnt signaling is low in human ESC and increases during their differentiation so that inhibiting Wnt signaling does not affect the cell self-renewal (33). In contrast, the Wnt signaling level is high in rESC, and inhibiting Wnt signaling causes cell differentiation (Table 1 and Fig. 1) similar to the case with mouse ESC (32). Although Wnt signaling plays different roles in rESC and hESC, BMP4 causes differentiation of both human (13, 51) and rabbit ESC. We also observed that BMP4 antagonizes the self-renewal-promoting effect of the TGFβ ligands and bFGF in rESC (Fig. 3). It remains to be explored, however,

FIGURE 4. **Self-renewal was supported by mixtures of TGFβ1/activin A/Nodal plus bFGF and Noggin in rESC.** *A*, RF line rESC were cultured on Matrigel in KSR medium alone at the first passage (*NC*, negative control) or in KSR medium supplemented with 10 ng/ml bFGF (*F*) and 10 ng/ml activin A (*A*), 100 ng/ml Nodal (*Nod*) or 1 ng/ml TGFβ1 (*T*) ± 100 ng/ml Noggin (*Nog*) for *in situ* detection for various markers. *F+A*, rESC cultured in bFGF + activin A for 4 days at the first passage (*p1*) and at passage 6 (*p6*); *F+A+Nog*, cultured at passage 15; *F+T+Nog*, cultured at passage 15; *F+Nod+Nog*, cultured at passage 15. The cell nuclei were counterstained with Hoechst 33342 (*blue*). Similar results were obtained in F+T and T+Nod at passages 1 and 6 as for those in F+A. RP01 cells grown in these culture systems had similar results to RF cells. *B*, RF line rESC cultured similarly as described above were subjected to Western blotting analysis for various markers. *Lane 1*, stands for F+A (at passage 1); *lane 2*, F+A+Nog (at passage 15); *lane 3*, F+Nod+Nog (at passage 15); *lane 4*, F+T+Nog (at passage 15); and *lane 5*, KSR medium alone (passage 1, as a negative control). Similar results were obtained from RP01 line rESC (not shown). *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase. *C* and *D*, RF and RP01 rESC cultured on Matrigel in KSR medium supplemented with F+A+Nog at passage 15 had normal karyotypes 44 XY and 44 XX, respectively. Similar results were obtained from F+T+Nog and F+Nod+Nog (not shown).

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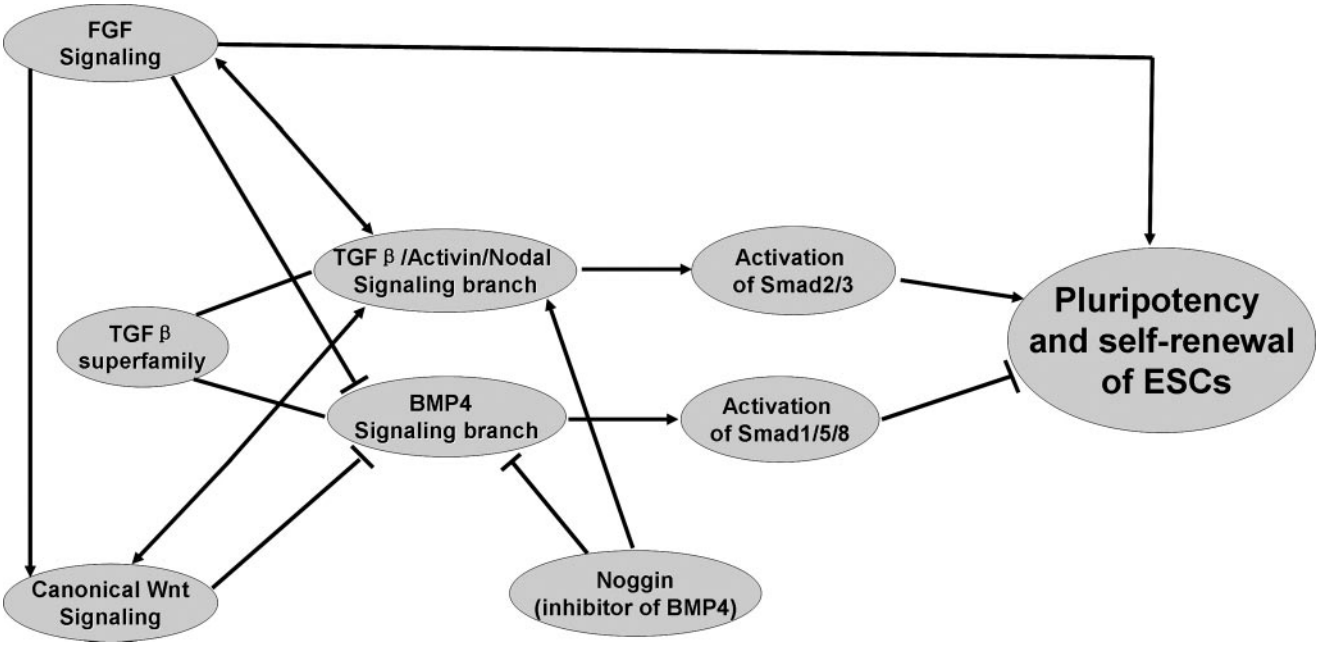

FIGURE 6. **A scheme illustrating the interactions among FGF, TGFβ, BMP, and Wnt signaling to sustain rESC self-renewal.** The *arrow-ended lines* denote stimulation, and the *hammer-ended lines* denote inhibition. Our results indicated that the FGF and TGFβ pathways cooperated to play essential roles in pluripotency and self-renewal of rESC, and activation of Smad2/3 signaling and inhibition of Smad1/5/8 signaling were necessary for pluripotency maintenance in rESC. These results imply that the effects of the FGF pathway on the maintenance of rESC pluripotency depend in part on the TGFβ pathway to activate Smad2/3 and inhibit Smad1/5/8, and at the same time, the FGF pathway also affects the stemness of rESC directly or indirectly through other pathways. The Wnt pathway also has an effect on rESC, which is weaker than that of the FGF and TGFβ pathways, and it is also suggested that the effect of the Wnt pathway on rESC might be mediated through the TGFβ pathway.

as to whether BMP4-treated rESC differentiate to trophoblasts, primitive endodermal, cells or any other lineages.[6]

Finally, the MAPK/ERK and PI3K/AKT pathways, both of which lie downstream of the FGF pathway, were involved in ES cell biology. In our study, MAPK/ERK and PI3K/AKT signaling activity were necessary for maintaining rESC in an undifferentiated state, and our results also suggested that both the MAPK/ERK and PI3K/AKT cascades were downstream targets of the FGF pathway in rESC. Recent reports also have indicated that these two cascades are important for the maintenance of pluripotency and viability in human ESC (2, 26–28). However, FGF stimulation of the ERK1/2 signaling cascade triggers the transition of mouse ESC from self-renewal to lineage commitment (29), and self-renewal of mouse ESC is enabled by the elimination of differentiation-inducing signaling from MAPK (30). Our results showed that PD98059 (to repress MAPK/ERK pathway) and LY294002 (to repress PI3K/AKT pathway) had no inhibitory effect on the phosphorylation of AKT or ERK1/2, respectively, suggesting that there was no cross-talk between these two pathways in rESC and that these two pathways regulate rESC self-renewal in a cooperative manner. However, in mouse ESC, PI3K/AKT signaling played an inhibitory role in regulating MAPK/ERK signaling, and inhibition of PI3K/AKT pathway led to a reduction in the ability of LIF to maintain self-renewal through augmenting LIF-induced phosphorylation of ERK (31).

In our study, we also observed interactions among the TGFβ, FGF, and Wnt pathways in rESC. Inhibition of the TGFβ, FGF, or Wnt pathway in rESC resulted in: 1) a reduction of the phosphorylation and nuclear localization of Smad2/3; 2) an increase in the phosphorylation and nuclear localization of Smad1/5/8; and 3) an increase in the phosphorylation of β-catenin and a decrease in its nuclear translocation. These results imply that there is a regulatory network among the TGFβ, FGF, and Wnt pathways that regulates the stemness of rESC. Previous reports have suggested that bFGF can induce human ESC to express TGFβ1, Nodal, and the BMP antagonists Noggin and Gremlin, which helps the cells to promote their own self-renewal by antagonizing the BMP pathway through Smad1/5/8 inhibition, subsequently suppressing differentiation (47, 48, 49, 52, 53). However, the previous studies also showed that differentiation occurred in hESC when bFGF was replaced *in vitro* with BMP inhibitors such as Noggin (47, 54), implying the existence of bFGF-mediated pathways in addition to BMP inhibitory signaling. Our findings also showed that a combination of SU5402 and SB431542 increased the inhibitory effect compared with their addition alone (Table 1). Furthermore, neither bFGF nor TGFβ1/activin A/Nodal alone is sufficient to maintain rESC self-renewal. These results suggest that the TGFβ and FGF pathways act independently of each other to maintain rESC self-renewal. However, the addition of SB431542 to KSR+bFGF culture conditions increased the differentiation rate, which indicates that SB431542 could restore the differentiation reduction of rESC caused by bFGF to some extent (Fig. 3). These results implied that the effect of the FGF pathway on rESC self-renewal is partially dependent on the TGFβ pathway to activate Smad2/3 and inhibit Smad1/5/8, and at the same time, the FGF pathway also affects the stemness of rESC directly or indirectly through other pathways. In a previous

[6] S. Wang, Y. Shen, X. Yuan, K. Chen, X. Guo, Y. Chen, Y. Niu, J. Li, R.-H. Xu, Y. Yan, Q. Zhou, and W. Ji, unpublished data.

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study, it was shown that activation of Wnt signaling maintains the undifferentiated state of both mESC and hESC (32), which possibly occurs via a Wnt-induced increase in expression of Nodal (46). In our experiments, the cell differentiation rate increased with a combination of SU5402 and Frizzled-1 compared with the addition of SU5402 alone. However, the addition of Frizzled-1 did not reinforce or rescue the inhibitory effect of SB431542 (Table 1), which suggests that the effect of the Wnt pathway on the stemness of rESC is mediated by the TGFβ pathway but not the FGF pathway. These results suggest that the FGF pathway synergizes with the TGFβ or Wnt pathways to sustain rESC self-renewal. Fig. 6 depicts a scheme attempting to accommodate these interactions. Further studies are necessary to address the molecular mechanism of how these signaling pathways interact to regulate rESC self-renewal and direct their lineage-specific differentiation.

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