Dissecting Signaling Pathways That Govern Self-renewal of Rabbit Embryonic Stem Cells

Shufen Wang1,2, Yi Shen1,2, Xiaohua Yuan1,2, Kai Chen1,2, Xiangyu Guo1,2, Yongchang Chen1,5, Yuyu Niu1,5, Jian Li3, Ren-He Xu4, Xiyun Yan2, Qi Zhou4,2,3, and Weizhi Ji2,1

From the 1Kunming Primate Research Center and Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming, Yunnan 650223, China, the 2Yunnan Key Laboratory of Animal Reproductive Biology, Kunming, Yunnan 650223, China, the 3Graduate School, Chinese Academy of Sciences, Beijing 100039, China, the 4National Laboratory of Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, China, **Department of Genetics and Developmental Biology and University of Connecticut Health Center for Regenerative Biology, University of Connecticut, Connecticut 06030-3301, and the 4Institute of Zoology, Chinese Academy of Sciences, Beijing 100108, China

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 primates 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

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.

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; Afp, alkaline phosphatase; pSmad, phospho-Smad; pCatenin, phospho-β-catenin; CMS, conditioned medium; EB, embryoid body; MEF, mouse embryonic fibroblast; FACS, fluorescence-activated cell sorting; SCID, severe combined immunodeficiency.

The Journal of Biological Chemistry 2008, 283(51) 35929–35940.

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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 (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 parthenogenetic 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 (10⁵/cm²) 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 × 10⁶ 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
Scientific) for 30 min on ice. 105 fixed (for Oct4) or live (for untreated cells) were fixed with 0.1% paraformaldehyde (Sigma-Aldrich) for 10 min at 4 °C and then permeabilized with 90% methanol (Fisher Scientific) for 8 min on ice. 106 cells were then incubated at 37 °C and then permeabilized with 0.1% paraformaldehyde (Sigma-Aldrich) for 10 min at 4 °C. The cell lysates were centrifuged at 12,000 × g for 5 min at 4 °C. Quantification of protein concentrations was carried out using the Protein Quantification Kit-Rapid (Bio-Rad) according to the manufacturer’s instructions.

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. Western Blotting

Cell expansion -fold after a treatment was calculated as the percentage of the 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 1 inhibitor > Frizzled-1 or anti-Wnt3a antibody

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.

RESULTS

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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.
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 FGF-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 SSEA-4+ 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 |
|-----------|------------------------------------------|--------------------------------|---------------------|
| Control (ESM) | 4.45 ± 0.21* | 88.14 ± 2.31* | 12.33 ± 1.17* |
| ESM + SU | 72.18 ± 0.97* | 44.72 ± 1.08* | 5.83 ± 0.44* |
| ESM + SB | 75.53 ± 2.20* | 51.57 ± 1.67* | 5.17 ± 0.44* |
| ESM + TI | 62.97 ± 2.07* | 60.25 ± 1.17* | 8.17 ± 1.07* |
| ESM + Fz | 38.34 ± 2.18* | 72.20 ± 1.03* | 9.67 ± 1.45* |
| ESM + anti-Wnt3a | 32.08 ± 1.94* | 69.62 ± 0.61* | 9.78 ± 1.03* |
| ESM + SU + Fz | 84.32 ± 1.22* | 32.54 ± 1.56* | 5.01 ± 0.78* |
| ESM + SB + Fz | 73.46 ± 1.09* | 39.69 ± 0.80* | 5.20 ± 0.65* |
| ESM + SU + SB | 96.52 ± 0.99* | 14.45 ± 0.59* | 2.01 ± 0.48* |
| ESM + 0.1% DMSO | 4.40 ± 0.23* | 86.66 ± 1.62* | 12.50 ± 0.88* |

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. 2C). 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).
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-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

![Figure 1](image_url)
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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. 4A). At later passages, the cells gradually differentiated, accompanied by the loss of the pluripotency markers (Fig. 4A). 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. 5A). 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. 5B).

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).
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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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 1 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,
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

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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.
Acknowledgments—We thank Drs. Yongtang Zheng and Yuqing Kuang for help and advice in Western blotting and Drs. Wenhui Nie and Jinhuai Wang for karyotype analysis of rESC. We also thank Dr. Barry Bavister for careful revision of the manuscript and Dr. Gen-Sheng Feng for critical reading of the manuscript.

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