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DEVELOPMENTAL BIOLOGY OF MAMMALS

Expression of TGFβ Family Factors and FGF2 in Mouse and Human Embryonic Stem Cells Maintained in Different Culture Systems

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Abstract—Mouse and human embryonic stem cells are in different states of pluripotency (naive/ground and primed states). Mechanisms of signaling regulation in cells with ground and primed states of pluripotency are considerably different. In order to understand the contribution of endogenous and exogenous factors in the maintenance of a metastable state of the cells in different phases of pluripotency, we examined the expression of TGFβ family factors (ActivinA, Nodal, Lefty1, TGFβ1, GDF3, BMP4) and FGF2 initiating the appropriate signaling pathways in mouse and human embryonic stem cells (mESCs, hESCs) and supporting feeder cells. Quantitative real-time PCR analysis of gene expression showed that the expression patterns of endogenous factors studied were considerably different in mESCs and hESCs. The most significant differences were found in the levels of endogenous expression of *TGFβ1*, *BMP4* and *ActivinA*. The sources of exogenous factors ActivnA, TGFβ1, and FGF2 for hESCs are feeder cells (mouse and human embryonic fibroblasts) expressing high levels of these factors, as well as low levels of BMP4. Thus, our data demonstrated that the in vitro maintenance of metastable state of undifferentiated pluripotent cells is achieved in mESCs and hESCs using different schemes of the regulations of ActivinA/Nodal/Lefty/Smad2/3 and BMP/Smad1/5/8 endogenous branches of TGFβ signaling. The requirement for exogenous stimulation or inhibition of these signaling pathways is due to different patterns of endogenous expression of TGFβ family factors and FGF2 in the mESCs and hESCs. For the hESCs, enhanced activity of ActivinA/Nodal/Lefty/Smad2/3 signaling by exogenous factor stimulation is necessary to mitigate the effects of BMP/Smad1/5/8 signaling pathways that promote cell differentiation into the extraembryonic structures. Significant differences in endogenous *FGF2* expression in the cells in the ground and primed states of pluripotency demonstrate diverse involvement of this factor in the regulation of the pluripotent cell self-renewal.

Keywords: embryonic stem cells, pluripotent, ground and primed states, differentiation, signaling pathways, TGFβ, ActivinA, FGF2

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INTRODUCTION

Mouse and human embryonic stem cells are in vitro models of pluripotent cells of mammal embryos, which are in different states of pluripotency (naive/ground and primed states) (Nichols and Smith, 2009). During ground-to-primed state transition, pluripotent embryonic cells lose the ability to give rise to germ cell line (Hayashi and Surani, 2009; Guo et al., 2009; Han et al., 2010). Mouse embryonic stem cells (mESCs), maintained in vitro in a ground state of pluripotency, are homologous with inner cell mass of blastocysts because they are able to develop into all types of somatic and germ cells after their injection in blastocyst. However, the self-renewal rates and cell culture systems for maintenance of the human embryonic stem cells (hESCs) and mESCs, both derived from blastocysts, are different (Smith et al., 1988; Savatier et al., 1996; Thomson et al., 1998; Burdon et al., 2002; Dahéron et al., 2004; Vallier et al., 2005; Xu et al., 2005; Becker et al., 2006). Moreover, hESCs have been found to be more similar to later embryonic population cultivated in vitro, mouse epiblast stem cells (EpiSCs) in the primed state of pluripotency (Brons et al., 2007; Tesar et al., 2007; Vallier et al., 2009; Hanna et al., 2010; Gordeeva et al., 2011).

Pluripotent mESCs and hESCs are maintained in different cell culture systems that can be considered as artificial cell niches ensuring the optimal microenvironment for self-renewal of the pluripotent cells. Mouse and human ESCs can grow on feeder cells derived from fibroblasts of different origin, as well as in feeder-free systems, which include diverse extracellular matrix proteins and defined sets of growth factors (Smith et al., 1988; Xu et al., 2001; Stojkovic et al., 2005; Vallier et al., 2005; Yoo et al., 2005; Eiselleova

et al., 2008; Evseenko et al., 2009; Montes et al., 2009; Koltsova et al., 2011, 2012). However, mESCs and hESCs require different sets of growth factors for self-renewal, which indicates diverse signaling pathways regulation of the ground and primed states of pluripotency, as well as determination of the early embryonic populations. Signaling pathways of TGFβ family factors and FGF2 are the key regulators of pluripotent state and in vivo and in vitro ESC differentiation (Mummery, 2001; Valdimarsdottir and Mummery, 2005; Dreesen and Brivanlou, 2007; Pucéat, 2007; Lanner and Rossant, 2010). Nevertheless, functional roles of these signaling pathways in cells in ground and primed states of pluripotency remain unclear.

In vitro modeling of the early mammal development using mESCs and hESCs allows studying the signaling regulations during development of mammal pluripotent cells and their differentiation into various cell types. In order to investigate the signaling pathways in the cells in different states of pluripotency, we examined the expression of TGFβ family factors and FGF2/bFGF initiating the appropriate signaling pathways in mESCs, hESCs and supporting feeder cells. According to data on comparative analysis of the expression of TGFβ and FGF2 family factors in mESCs and hESCs, we have hypothesized the functional roles of signaling pathways initiated by these factors in regulation of ground-to-primed states transition of pluripotent cells and differentiation of early embryonic cell populations.

MATERIALS AND METHODS

In vitro cell culture. Mouse ESC R1 line provided by Dr. A. McLaren (WTCR Institute of Cancer and Developmental Biology, Cambridge, United Kingdom) was used. Human ESC ESM02 line was kindly provided by Prof. G.P. Georgiev (Institute of Gene Biology, Russian Academy of Sciences, Moscow, Russia). Human hESC SC5 and human embryonic fibroblast lines were derived and characterized earlier in Cell Culture Department of Institute of Cytology, Russian Academy of Sciences, St. Petersburg, Russia) Koltsova et al., 2011, 2012).

Mouse and human ESCs were cultivated in Dulbecco's modified Eagle medium (DMEM) supplemented with 1 mM L-glutamine, 0.1 mM nonessential amino acids (HyClone, United States), 0.1 mM β-mercaptoethanol (Sigma, United States), and 15% Characterized Fetal Bovine Serum (Knockout Serum Replacement, Gibco, United States). Undifferentiated mESs were maintained on mouse embryonic fibroblast feeder cells (mEFs, derived from E12.5 embryos of C57B1/6 mouse strain) inactivated by mitomycin C treatment (10 μg/mL, Sigma, United States) or in a feeder-free system in media containing leukemia inhibitory factor (LIF, 10 ng/mL, Sigma, United States). Undifferentiated hESC ESM02 line was maintained on inactivated feeder cells derived from mEFs, while hESCs SC5 line was maintained on feeder cells derived from hEFs. Culture medium for hESCs was supplemented with recombinant fibroblast growth factor (FGF2/bFGF, 10 ng/mL, Invitrogen, United States).

Mouse and human EFs were cultivated in DMEM supplemented with 1 mM L-glutamine (HyClone, United States) and 10% Characterized Fetal Bovine Serum (HyClone, United States). For analysis of gene expression, feeder cells were cultivated in mES and hES cell media for 24 hours.

Feeder cells, mEFs and hEFs, as well as mESCs, were subcultured using 0.05% Trypsin-EDTA solution (HyClone, United States). Colonies of undifferentiated hES cells were manually divided into cell clusters for their propagation.

Embryoid bodies (EB) formed during differentiation of the mESCs were generated using the "hanging drops" method as described (Gordeeva et al., 2009). EBs of ESM02 and SC5 lines were obtained by cultivation of cell clusters of manually divided colonies of undifferentiated hESCs in low adhesion culture plates(Greinerbio, Germany) for 5 days.

Gene expression analysis. Gene expression was analyzed in cells and EBs grown in serum-free medium: in mEFs and hEFs, in mESCs maintained in LIF containing media, in hESCs manually isolated from feeder, and in EBs after 5 days of cultivation in LIF-free and bFGF-free media. Total RNA was extracted from all cells using TRIzol Reagent (Invitrogen, United States) according to the manufacturer's recommendations. The total RNA concentrations in the samples were evaluated using a NanoDrop 8000 spectrophotometer (ThermoScientific, United States). To avoid DNA contamination all samples of total RNA were treated with TurboDNasa (Ambion, United States) according to manufacturer protocols. One microgram of total RNA from each sample was reverse transcribed using M-MuLV revertase and $(dT)_{18}$ oligonucleotide primers (Fermentas, Lithuania) for cDNA synthesis.

Quantitative analysis of gene expression was performed in an Applied Biosystems 7500 cycler (United States) using RT-PCR kit with the EVA Green Dye (Sintol, Russia) according to the following protocol: initial) denaturation at 94°C for 5 min; annealing of primers and elongation at 62°C for 45 s. 40 cycles of denaturation at 94°C for 15 s. Fluorescence level was detected at 62°C in every cycle. Specific primers were designed on the basis of structure of studied genes from GenBank, MGI, and Ensemble databases (Tables 1, 2). For all used primer pairs, the doubling of product quantity in each amplification cycle, as well as the synthesis of the only amplicon of expected product, were confirmed. All experiments were run in triplicate. Level of gene expression in each sample was normalized to the level of expression of hypoxanthine guanine phosphoribosyl transferase (*HPRT/Hprt*) gene. Relative level of mRNA was calculated using the

Table 1. Structure of the primers used for analysis of gene expression in mouse embryonic stem cells and embryonic fibroblasts

Gene	No. of sequence	Forward and reverse primers	Size, bp
Oct4/Pou5f1	NM_013633.2	5'caccctgggcgttctctttg3'	142
		5'gttctcattgttgtcggcttcc3'	
Nanog	NM_028016	5'aactctcctccattctgaacctga3'	136
		5'ggtgctgagcccttctgaatc3'	
Gata4	NM_008092	5'tctcactatgggcacagcag3'	100
		5'gggacagcttcagagcagac3'	
ActivinA	NM_002192	5'tggagcagacctcggagatcatcac3'	160
		5'ttggtcctggttctgttagccttgg3'	
Nodal	NM_013611	5'gcgagtgtcctaaccctgtg3'	136
		5'atgctcagtggcttggtc3'	
Lefty1	NM_010094	5'tgtgtgctctttgcttcctctg3'	123
		5'gcagtgaacaatatgaaggacagag3'	
Tgfβ1	NM_011577	5'caattcctggcgttaccttgg3'	120
		5'ccctgtattccgtctccttgg3'	
Bmp4	NM_007554	5'tctggtctccgtccctgatg3'	175
		5'cgctccgaatggcactacg3'	
Gdf3	NM_008108	5'gatgagtgtgggtgtgggtag3'	109
		5'gtccgattcaagagagcataagc3'	
Fgf2	NM_008006	5'cgtcaaactacaactccaagcag3'	147
		5'tccagtcgttcaaagaagaaacac3'	
Hprt	NM_013556	5'cgttgggcttacctcactgctttc3'	150
		5'ggtcataacctggttcatcatcgctaatc3'	

comparative Ct-method (ABI Relative Quantification Study Software, Applied Biosystems, United States). Relative levels of gene expression were calculated according to the formulae $2^{-\Delta\Delta Ct} \pm$ SD. The gene expression level in undifferentiated ESCs or untreated fibroblasts served as a relative unit. In analysis of expression levels of TGFβ family factors and FGF2 in all cell types, *HPRT* gene expression level served as a relative unit, while the expression level of all other genes in a sample was calculated according to the formulae $2^{-\Delta Ct} \pm$ SE.

RESULTS

Endogenous expression of TGFβ family factors and FGF2 in undifferentiated and differentiating mouse and human ESCs. For analysis of the role of signaling pathways of TGFβ family factors and FGF2 in the regulation of pluripotent states of mouse and human ESCs, we examined the expression of these signaling factors and specific transcription factors OCT4/Oct4, NANOG/Nanog, and GATA4/Gata4 in undifferentiated and differentiating cells (Figs. 1, 2). According to data on quantitative PCR-analysis (Fig. 1b) during differentiation of EBs formed by ESM02 and SC5 cells, the expression of *OCT4* gene decreased 4.4 and 2.2 times, while the expression of *NANOG* decreased 3.1 and 13.5 times, respectively. At the same time, the expression of *Oct4* and *Nanog* decreased only 1.1–1.2 times in EBs formed by mESCs (Fig. 1b). The differentiation of ESCs into extraembryonic endoderm followed by the increase in expression of *GATA4* by 737, 58, and 16 times in EBs of ESM02, SC5, and R1 cell lines, respectively. Thus, our data demonstrated that hESCs initiate the differentiation faster and more effective, than mESCs.

Expression analysis of TGFβ family factors and FGF2/bFGF showed that all factors expressed in undifferentiated mESCs and hESCs, although expression levels of most factors had decreased during EB differentiation (Fig. 2). The largest decline of gene expression levels in differentiating human EBs was found for *LEFTY1* (200 and 16 times for ESM02 and SC5, respectively), while that in mouse EBs was found for ActivinA gene (83 times). Gene expression of *TGFβ* and *BMP4* hardly changed in all ESC lines. It should be noted that the decrease in the expression of genes *ACTIVINA/ActivinA*, *NODAL/Nodal*,

Table 2. Structure of the primers used for analysis of gene expression in human embryonic stem cells and embryonic fibroblasts

Gene	No. of sequence	Forward and reverse primers	Size, bp
OCT4/POU5F1	NM_002701	5'cgaaagagaaagcgaaccagtatc3'	220
	NM_203289	5'acccagcagcctcaaaatcc3'	
NANOG	NM_024865	5'caagaactctccaacatcctgaacc3'	127
		5'tctgcgtcacaccattgctattc3'	
GATA4	NM_002052	5'gatgggacgggtcactatctg3'	160
		5'ggcagttggcacaggagag3'	
ACTIVINA	NM_002192	5'agggcagaaatgaatgaacttatgg5'	198
		5'gaggcggatggtgactttgg5'	
NODAL	NM_018055	5'tcaactgtgtcggaaggtcaag3'	190
		5'tcggtggggctggtaacg3'	
LEFTY1	NM_020997	5'tcattgtttacttgtcctgtcactg3'	116
		5'agtctttattatctggattggggatgc3'	
TGFβ1	NM_000660	5'tggacatcaacgggttcactac3'	186
		5'gcacgcagcagttcttctcc3'	
BMP4	NM_001202	5'tgagtgccatctccatgctgta3'	91
	NM_130850	5'cggcacccacatccctctacta3'	
	NM_130851		
GDF3	NM_020634	5'cttcgctttctcccagaccaag3'	128
		5'gccaatgtcaactgttccctttc3'	
FGF2	NM_002006	5'gccagtaatcttccatcttcccttc3'	113
		5'tgtgtgctctttgcttcctctg3'	
HPRT	NM_000194.2	5'aagatggtcaaggtcgcaagc3'	132
		5'gaagtattcattatagtcaagggcatatcc3'	

LEFTY1/Lefty1, *GDF3/Gdf3*, and *FGF2/Fgf2* correlated with the changes in the expression of *OCT4/Oct4*, *NANOG/Nanog*, and *GATA4/Gata4* during the differentiation of mouse and human EBs. In addition, the expression of genes *NODAL/Nodal*, *LEFTY1/Lefty1*, *GDF3/Gdf3*, and *FGF2/Fgf2* decreased considerably stronger in hESCs. These findings indicate that endogenous TGFβ and FGF2 family factors are involved in regulation of pluripotent state and differentiation of mESCs and hESCs. However, the effects of signaling pathways initiated by these factors are probably different in hESCs and mESCs, which results in their different differentiation rate.

Expression analysis of TGFβ family factors and FGF2 in feeder cells mEFs and hEFs, used for the maintenance of undifferentiated mESCs and hESCs. For analysis of the role of signaling pathways initiated by TGFβ family factors and FGF2 in the maintenance of undifferentiated state of mESCs and hESCs, we examined the expression of these factors in feeder cells. Feeder cells are the source of extracellular matrix proteins and the exogenous factors of TGFβ family and FGF2 involved in the general regulation system for the maintenance of undifferentiated mESCs and hESCs as well. Mouse and human TGFβ family factors are characterized by up to 90% of homology; thus, they can be utilized by the cells of both species. Mouse ESCs R1 and human ESCs ESM02 were maintained on feeder cells mEFs, while human ESCs SC5 was cultivated on feeder cells hEFs (Fig. 3). Both feeder systems were effective in the maintenance of the growth of undifferentiated mESCs and hESCs. However, to maintain the self-renewal of hESCs grown on both mEFs and hEFs, the cultivation medium was supplemented with FGF2/bFGF factor.

Investigation of the gene expression of TGFβ family factors and FGF2 in mEFs and hEFs showed that both types of feeder cells had a similar expression pattern: high level of *ACTIVINA/ActivinA*, *TGFβ1/Tgfβ1*, and *FGF2/Fgf2*, as well as low level of *NODAL/Nodal*, *LEFTY1/Lefty1*, *GDF3/Gdf3*, and *BMP4/Bmp4* (Fig. 4). Expression analysis of the factors in mEFs

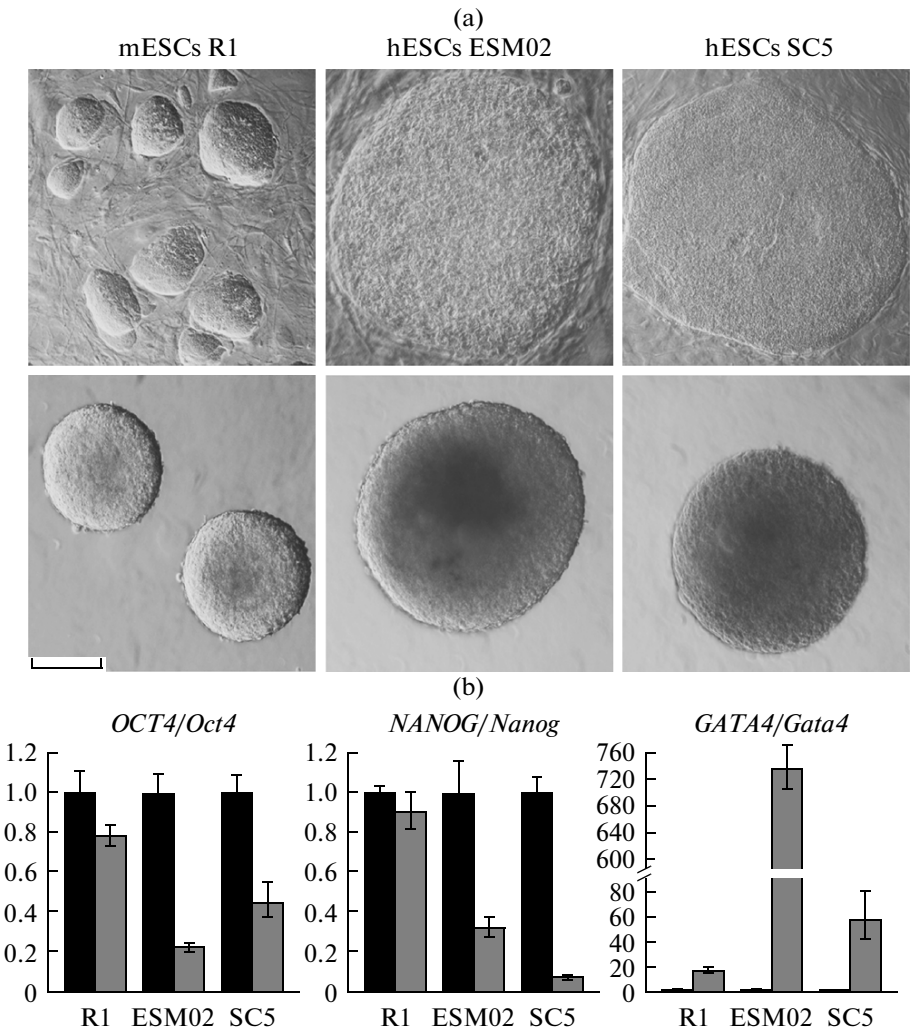

Fig. 1. (a) Colonies of undifferentiated mouse and human ESCs cultivated on mouse embryonic fibroblasts and human embryonic fibroblasts (upper series), and embryonic bodies formed by these cells during differentiation (lower series). Scale: 100 μm; (b) Quantitative analysis of expression of the genes *OCT4/Oct4*, *NANOG/Nanog*, and *GATA4/Gata4* in mouse and human ESCs. The relative level of gene expression normalized to expression level of *HPRT/Hprt* is indicated on the *y*-axis. Expression level in undifferentiated cells served as 1 relative unit. Black column—undifferentiated cells; gray column—EB5.

and hEFs before and after mitomycin C treatment and FGF2/bFGF addition showed that expression of *ACTIVINA/ActivinA*, *TGFβ1/Tgfβ1*, and *FGF2/bFGF* remained at a high level, while low expressed factors varied insignificantly (Fig. 4). However, correlation between levels of the expression of *ACTIVINA/ActivinA*, *TGFβ1/Tgfβ1*, and *FGF2/Fgf2* relative to endogenous expression of *HPRT/Hprt* gene in both feeder systems showed that all three genes were characterized by significantly higher expression levels in human EFs compared with mEFs (Figs. 4b, 4d). In addition, comparative analysis demonstrated that the expression levels of *HPRT/Hprt* were not considerably different in mEFs and hEFs. For cDNA samples synthesized from 1 μg of total RNA, the values of threshold cycle Ct of *HPRT/Hprt* were 22.907 ± 0.038 and 22.967 ± 0.099 for mEFs and hEFs, respectively. Thus, feeder cells mEFs and hEFs expressing different levels of TGFβ family factors and FGF2 formed various niches for the self-renewal of the pluripotent mESCs and hESCs.

Analysis of interactions between endogenous and exogenous signaling pathways of TGFβ family factors and FGF2 and the regulation of the pluripotent states in mESCs and hESCs.

According to our findings on the expression patterns of TGFβ family factors and *FGF2* in ESCs and feeder cells, we analyzed the correlation between endogenous and exogenous signaling supporting the in vitro maintenance of mESCs and hESCs in an undifferentiated state (Fig. 5). Calculation the ratio of the expression levels of the studied factors to the *HPRT/Hprt* expression, we revealed that the highest level of expression was found for *Lefty1* gene in mESCs (six times more than *Hprt* level). The expression levels

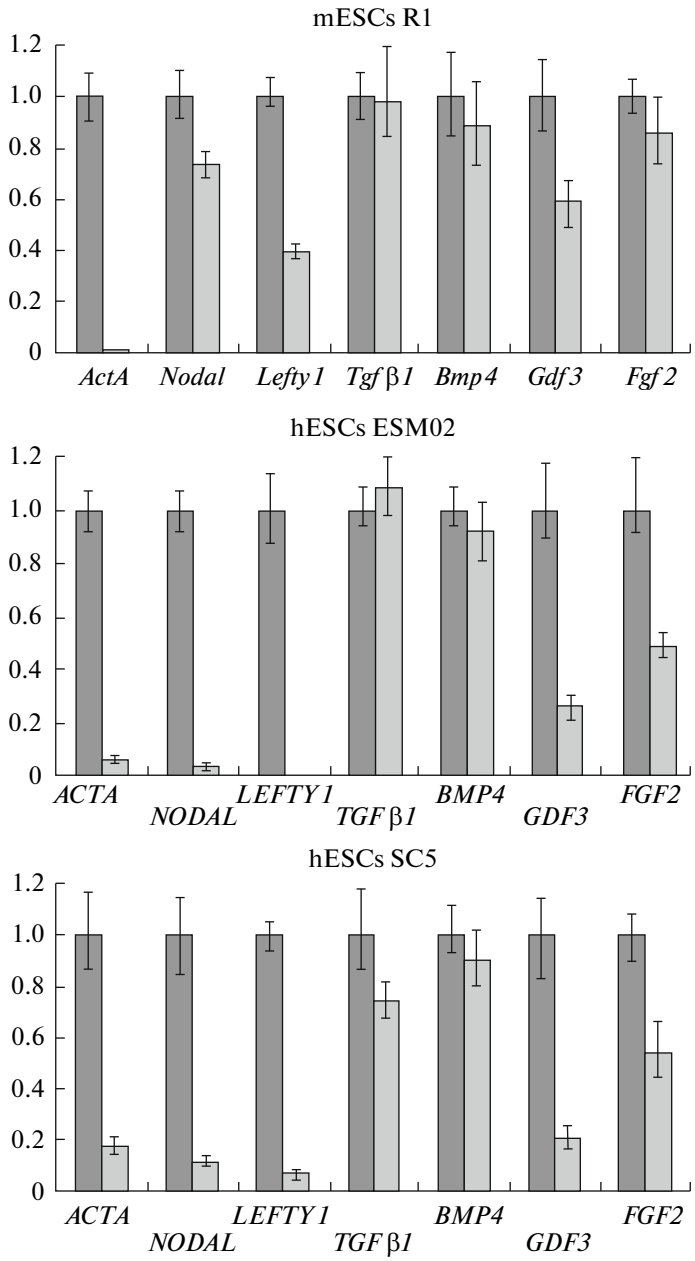

Fig. 2. Quantitative analysis of the expression of TGFβ family factors and FGF2 during differentiation of mouse and human ESCs. The relative level of gene expression normalized to expression level of *HPRT/Hprt* is indicated on the *y*-axis. Expression level in undifferentiated cells served as 1 relative unit. Dark column—undifferentiated cells; light column—EB5.

of *ActivinA*, *Tgfβ1*, and *Bmp4* genes were comparable to the expression level of *Hprt* gene, while *Nodal*, *Gdf3*, and *Fgf2* expressed at a lower level than *Hprt*.

Expression patterns of studied factors were considerably different between hESCs and mESCs lines (Fig. 5). The most considerable differences were found in the expression patterns of *TGFβ1* and *BMP4* genes, and besides the expression levels of these genes were also two times different for ESM02 and SC5. In both hESCs lines, the highest expression levels were revealed for *TGFβ1* and *BMP4* genes (10–20 times more than in *HPRT*); the expression level of *LEFTY1* was 3–6 times more than in *HPRT*, but it was comparable to the that in mESCs. The expression patterns of *NODAL* and *GDF3* in ESM02 and SC5 cells was 2–3 times more than the expression levels of *HPRT* in these lines. In this case, ratios of the expression levels between these genes and *HPRT/Hprt* were 2–3 times more for *NODAL* and 10 times more for *GDF3* in hESCs compared with mESCs. In both hESC lines, similar expression levels of *ACTIVINA* and *FGF2* genes were found, which were lower than *HPRT* level. How-

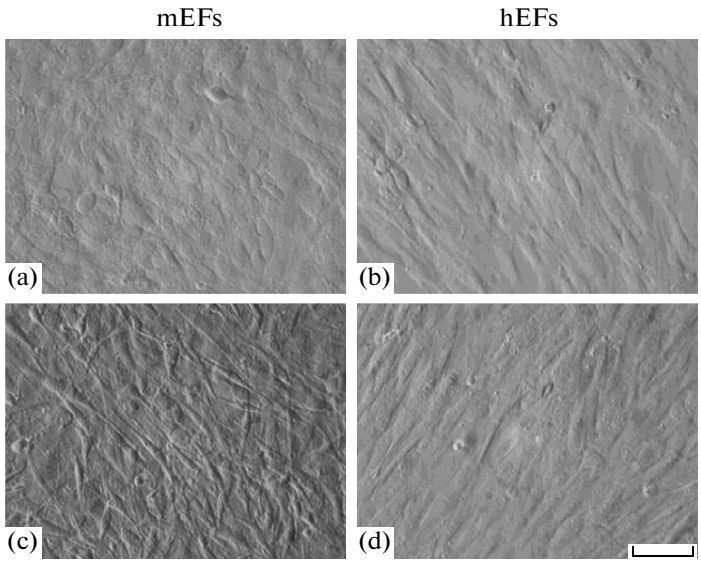

Fig. 3. Feeder cells mEFs and hEFs (a), (b) before and (c), (d) after mitomicin C and FGF2 treatment. Scale: 100 μm.

ever, the expression of *ACTIVINA* in hESCs was lower than in mESCs, while it was more than 10 fold higher for *FGF2/Fgf2*. Thus, the expression levels of endogenous TGFβ family factors and FGF2 were different in the pluripotent mESCs and hESCs. At the same time, the endogenous expression patterns of studied signaling ligands were similar in both hESC lines maintained on different feeder cells.

Correlative analysis between the expression levels of TGFβ family factors and FGF2 in mESCs R1 and hESCs SC5 and their feeder cells showed that the expression levels of *ActivinA/ACTIVINA* in mEFs and hEFs were 4 and 42 fold higher than the expression levels in mESCs and hESCs, respectively (Fig. 6). At the same time, the expression levels of *Tgfβ1/TGFβ1* and *Fgf2/FGF2* were 5 and 30/16 times more in mEFs and hEFs than in mESCs and hESCs (Fig. 6). Considering the fact that the maintenance of undifferentiated mESCs is possible in a feeder-free system supplemented with the LIF and without the exogenous ActivinA, TGFβ1, and FGF2, probably, the levels of endogenous expression of these factors are optimal for the invariance of the pluripotent state of mESCs in the vitro culture. In addition, ActivinA, TGFβ1, and FGF2 produced by mEFs have no significant effect on self-renewal and differentiation of mESCs grown on this feeder cells.

On the contrary, preservation of the defined levels of both endogenous and exogenous (feeder cells and recombinant factors) *ACTIVINA*1, *TGβ1*, *BMP4* and *FGF2* in a medium is critical for in vitro maintenance of undifferentiated state of hESCs. Obviously, feeder cells together with recombinant FGF2 produce the optimal quantity of ACTIVINA and FGF2 factors necessary for the maintenance of undifferentiated hESCs in vitro. In addition, BMP4 factor stimulating differentiation of hESCs express at a low level in both mEFs and hEFs.

DISCUSSION

Pluripotent cells of inner cell mass and epiblast at preimplantation developmental stages are surrounded by the cells of two extraembryonic structures—trophoblast and hypoblast (primary extraembryonic endoderm), which are natural niches for embryonic cells with ground state of pluripotency. Cells of trophoblast and hypoblast express factors essential for further development of pluripotent cells, including their transition to the next phase—primed state of pluripotency. Pluripotent cells of inner cell mass transferred on feeder cells in vitro get into another microenvironment that stimulates their proliferation and inhibits the differentiation. It is possible to maintain mouse and human ESCs on mEFs, but the maintenance of undifferentiated hESCs requires the supplement of recombinant FGF2 in medium. Moreover, under feeder-free culture conditions mESC self-renewal is supported by LIF only while hESCs and mouse epiblast stem cells require a more complex culture system containing TGFβ family factors (ActivinA and Nodal) and FGF2, but not LIF (Smith et al., 1988; Thomson et al., 1998; Dahéron et al., 2004; Vallier et al., 2005; Xu et al., 2005; Brons et al., 2007; Tesar et al., 2007; Vallier et al., 2009). However, compared with mESCs, the part of hESC population comes into spontaneous differentiation using both feeder and feeder-free cultivation. Thus, the maintenance of self-renewal of the cells in ground and primed states of pluripotency is provided by various signaling pathways and different functional activity of LIF/Stat3,

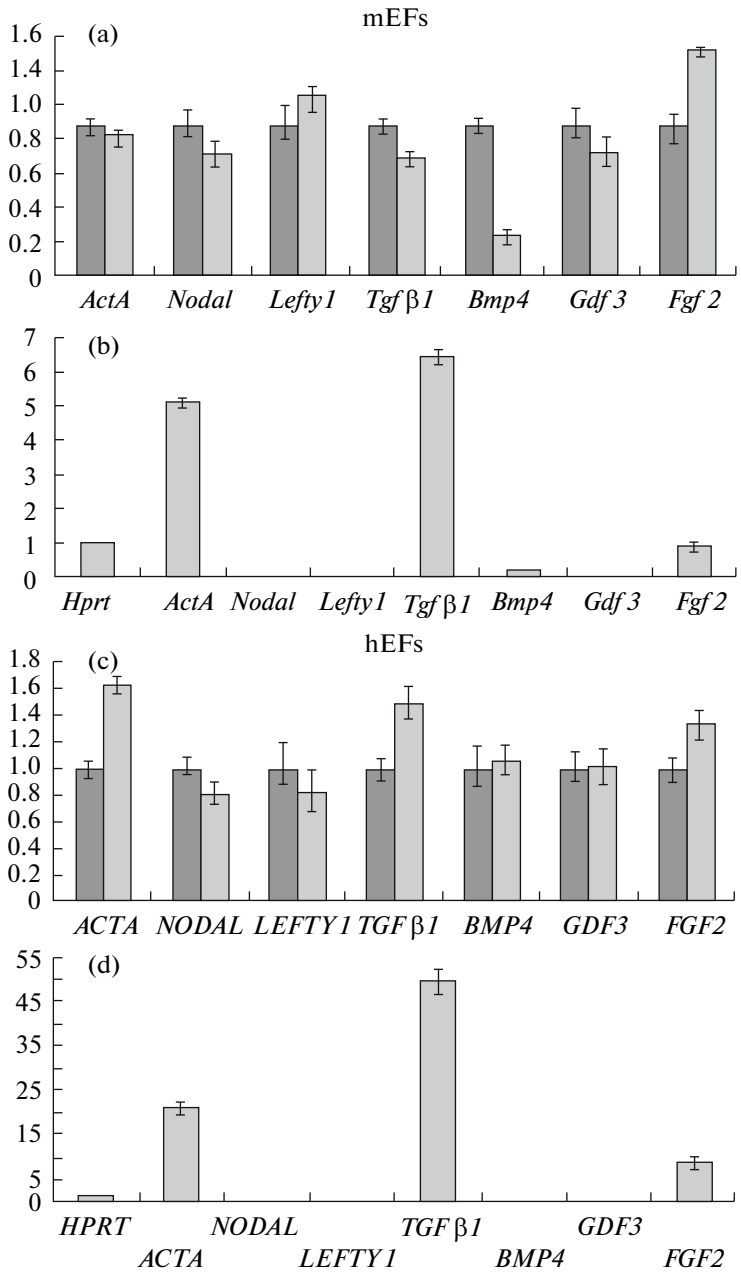

Fig. 4. Analysis of expression of TGFβ family factors and FGF2 in mEFs and hEFs. The relative level of gene expression normalized to expression level of *HPRT/Hprt* is indicated on the *y*-axis. (a), (c) Expression level in untreated cells served as 1 relative unit or (b), (d) expression level of gene *HPRT/Hprt* in treated cells served as 1 relative unit; dark column—undifferentiated cells; light column—cells treated with mitomicin C and FGF2.

Activin/Nodal/TGFβSmad2/3, and FGF2 signaling pathways.

To investigate the signaling pathways regulating the self-renewal of pluripotent stem cells in different phases of pluripotency we analyzed the correlation between endogenous and exogenous expression of the signaling ligands of TGFβ family and bFGF in mESCs, hESCs and their feeder cells. Firstly, we found that the patterns of endogenous expression of these factors were considerably different in mESCs and hESCs (Fig. 5). Thus, in mESCs the highest expression level was found for *Lefty1* gene, while in hESCs that for *TGFβ1* and *BMP4* was found. In addition, the expression of *NODAL/Nodal*, *TGFβ1/Tgfβ1*, *BMP4/Bmp4*, *GDF3/Gdf3*, and *FGF2/Fgf2* was higher in hESCs than in mESCs. At the same time, the expression of *ACTIVINA* was lower in both hESC lines than in mESCs. Thus, differences in the patterns of endogenous expression of studied signaling factors in undifferentiated mESCs and hESCs demonstrate

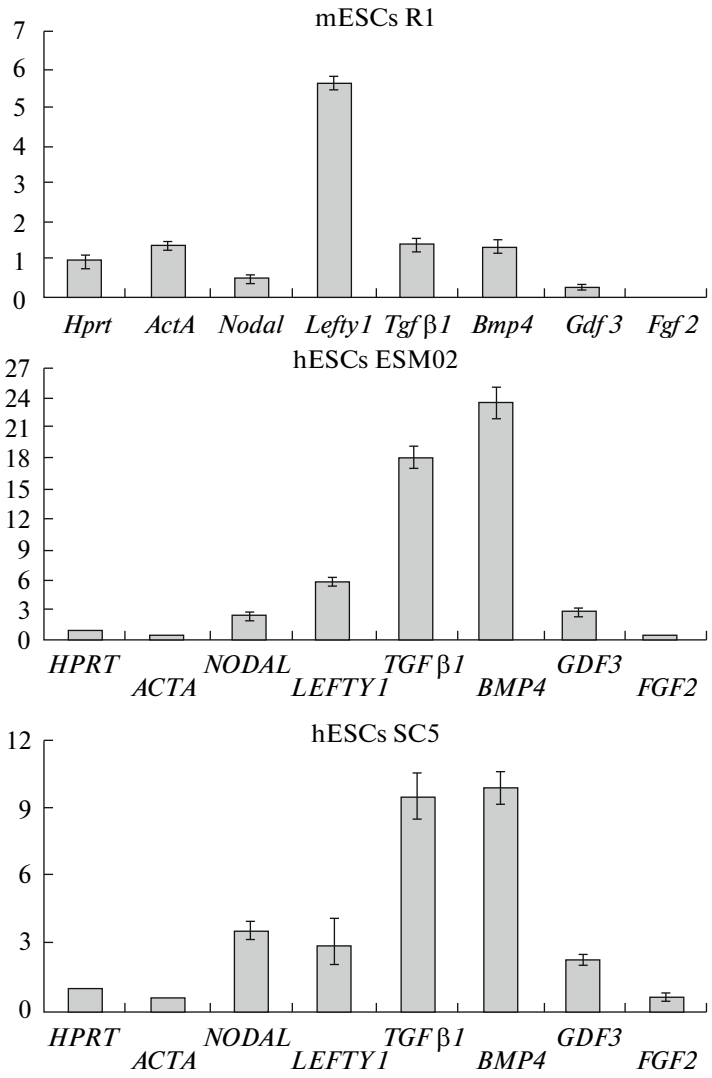

Fig. 5. Expressions of TGFβ family factors and FGF2 in undifferentiated mESCs and hESCs. The relative level of gene expression normalized to expression level of *HPRT/Hprt* is indicated on the *y*-axis. Expression level of *HPRT/Hprt* served as 1 relative unit.

internal diversity in signaling regulation of these cell populations. Furthermore, the higher EB differentiation rate of hESCs compared with mESCs was accompanied by more dynamic down-regulation of the factors *NODAL/Nodal*, *LEFTY1/Lefty1*, *GDF3/Gdf3*, and *FGF2/Fgf2*. We found similar decrease in the expression levels of these genes only at day 10 of differentiation of EBs derived from mESCs (unpublished data). Nevertheless, the expression levels of *TGFβ1/Tgfβ1* and *BMP4/Bmp4* varied insignificantly during the spontaneous differentiation of all ESC lines and they were the highest compared with other factors in EBs. Based on these data, we can suggest that the highest expression level of *TGFβ1/Tgfβ1* and *BMP4/Bmp4* against relatively lower expression level of other factors characterizes more differentiated ESC state. In this case, the tendency to spontaneous differentiation of hESCs compared with mESCs may be explained by higher expression level of *TGFβ1/Tgfβ1* and *BMP4/Bmp4* in the undifferentiated hESCs, which are at a more advanced developmental stage than mESCs. Another characteristic of signaling regulation in the undifferentiated hESCs is the lower level of the endogenous expression of *ACTIVINA*, which also dramatically decreases during the differentiation of mESCs and hESCs.

Expression analysis of TGFβ and FGF2 in feeder cells of mEFs and hEFs showed that they are a source of exogenous ActivinA, TGFβ1, and FGF2 for ESCs. These factors expressed at a higher level than in ESCs, although their expression level was significantly lower in mEFs than in hEFs. As noted above, exogenous ActivinA, TGFβ1, and FGF2 are not required for the mESC self-renewal in vitro, while exogenous factors ActivinA and FGF2 are necessary for hESCs. Both feeder systems used are capable of effectively maintain-

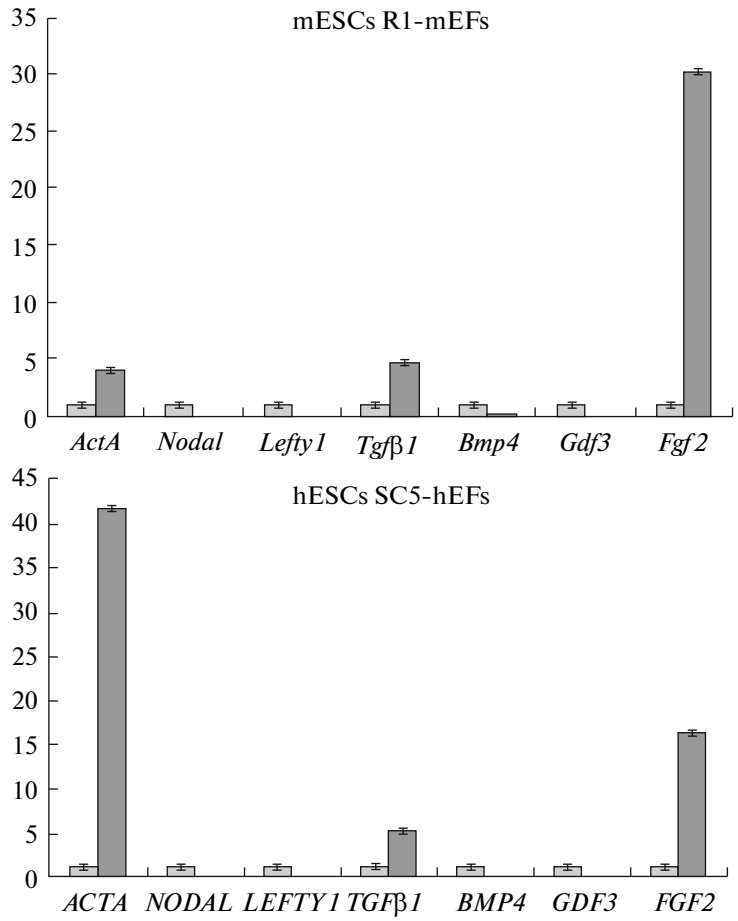

Fig. 6. Correlation between endogenous and exogenous expressions of TGFβ family factors FGF2 in undifferentiated mESCs and hESCs and supporting feeder cells. The relative level of gene expression normalized to expression level of *HPRT/Hprt* is indicated on the *y*-axis (expression level in undifferentiated cells served as 1 relative unit). Light column—undifferentiated mESCs R1 and hESCs SC5; dark column—mEFs and hEFs.

ing the self-renewal of hESCs in spite of significant differences in expression levels of *ACTIVINA/ActivinA* and *TGFβ1/Tgfβ1*, therefore expression levels of the factors can be acceptable for the maintenance of hESCs in these limits. However, the maintenance of hESCs on mEFs requires the supplement of the exogenous recombinant factor FGF2 because *Fgf2* expresses at a low level in these feeder cells. On the other hand, the supplement of the recombinant factor FGF2 is not necessary using hEFs or medium conditioned with hEFs. The properties of media conditioned with hEF and embryonic fibroblasts derived from hESCs of SC5 and SC7 lines to maintain the self-renewal of these hESC lines was shown previously (Koltsova et al., 2012). Feeder cells used for hESC maintenance may significantly differ in the expression levels of ActivinA, TGFβ1, and FGF2 factors, but these cultivation systems can be modified using exogenous recombinant factors (Eiselleova et al., 2008).

It should be noted that the expression level of *BMP4/Bmp4* was significantly lower in both types of feeder cells than in undifferentiated ESCs grown on them. Previously, it was shown that BMP4 together with LIF was able to maintain mESC self-renewal, although BMP4 was not indispensable factor (Ying et al., 2003). On the contrary, the exogenous BMP4 initiated the differentiation of hESCs in the cells of extraembryonic structures in hESC, while kinase inhibition of BMP receptors resulted self-renewal (Xu et al., 2002, 2005; Vallier et al., 2005, 2009; Greber et al., 2007). Effects of BMP/Smad1/5/8 signaling pathways to the differentiation of mESCs and hESCs may vary due to higher level of endogenous BMP4 in hESCs compared with mESCs. Considering that the increase of BMP/Smad1/5/8 branch of TGFβ signaling causes initiation of the hESC differentiation, the self-renewal of undifferentiated hESCs may be possible only on feeder cells with low expression level of BMP factors.

For maintenance of the metastable undifferentiated state of pluripotent ESCs in vitro the balanced activity of different signaling pathways, including ActivinA/Nodal/Lefty/Smad2/3 and BMP/Smad1/5/8 branches of TGFβ signaling, is required (Xiao et al.,

2006; Dreesen and Brivanlou, 2007; Greber et al., 2007). Apparently, high level of endogenous expression of *TGFβ1* in hESCs is not sufficient to neutralize the effects of endogenous BMP4 initiating the differentiation; thus, the increase in activity of ActivinA/Nodal/Smad2/3 branch using exogenous factors ActivinA and Nodal is required for the maintenance of signaling balance. Requirement of ActivinA/Nodal/TGFβ/Smad2/3 signaling for the proliferation of pluripotent cells of mouse blastocyst and maintenance of undifferentiated hESCs was demonstrated by inhibiting kinase receptor ALK-4, -5, -7 using inhibitor SB 431542 and after treatment with growth factors mentioned (Dunn et al., 2004; James et al., 2005; Vallier et al., 2005). However, differentiation of hESCs may also be initiated by high concentration of these factors in the medium (McLean et al., 2007).

The role of FGF2 factor in the self-renewal of hESCs remains unclear because of controversial data on the interaction between FGF2 and ActivinA signaling pathways. Previously, it was shown that FGF2 initiated the activity PI3K and ERK signaling pathways and in cooperation with ActivinA/Nodal signaling maintained the self-renewal and viability of hESCs (Vallier et al., 2005; Eiselleova et al., 2009). Besides, only FGF2 is not able to block the differentiation and to stimulate hESC proliferation (Vallier et al., 2005; Greber et al., 2007; Na et al., 2010). On the other hand, it was shown in a series of papers that exogenous ActivinA was sufficient for hESC maintenance in an undifferentiated state (Beattie et al., 2005; Xiao et al., 2006). In fact, hESCs differentiated faster after the supplement of ActivinA together with FGF2 (Na et al., 2010). In our experiments, hESCs ESM02 and SC5 cultivated on feeder cells expressing different levels of FGF2 remained undifferentiated in the case of high concentration of exogenous FGF2 (on mEFs only in the medium with recombinant FGF2).

Mouse epiblast stem cells, like hESCs, are cultivated in vitro in the medium with exogenous ActivinA and FGF2 (Brons et al., 2007; Tesar et al., 2007; Vallier et al., 2009). Reversion from primed to ground state of pluripotency in these cells (into mESC-like) is possible under 2i+LIF conditions—in the medium containing LIF and ERK1/2 (PD0325901) and GSK3-kinase (CHIR99021) inhibitors (Silva et al., 2008; Ying et al., 2008). Transition of hESCs from primed to ground states of pluripotency occurred during cultivation under 3i+LIF conditions—PD0325901/CHIR99021/Forskolin/LIF (Hanna et al., 2010). In both cases, the inhibition of ERK1/2 signaling cascades resulted in the lower dependence from exogenous factors ActivinA and FGF2.

Thus, during the transition of pluripotent cells from ground to primed states and to differentiation of early embryonic populations functional roles of signaling pathways initiated by TGFβ family factors and FGF2 change. Our findings suggest that the maintenance of metastable undifferentiated state of pluripotent cells in vitro ensured by balanced activity of different signaling pathways is achieved by different regulations of ActivinA/Nodal/Lefty/Smad2/3 and BMP/Smad1/5/8 branches of TGFβ signaling in mESCs and hESCs. Requirement of exogenous stimulation and inhibition of these signaling pathways is caused by internal differences in the expression pattern of TGFβ family factors and FGF2 in mESCs and hESCs. In hESCs, more advanced embryonic population, the enhancement of ActivinA/Nodal/Lefty/Smad2/3 signaling by exogenous factors stimulations is necessary to mitigate the effects of BMP/Smad1/5/8 signaling pathways promoting differentiation into the extraembryonic structures. Various modulating effects of FGF2 in the cells in ground and primed states of pluripotency are obviously caused by different functional activity of ERK1/2 signaling cascades.

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