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ORIGINAL ARTICLE

Derivation and transcriptional profiling analysis of pluripotent stem cell lines from rat blastocysts

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Embryonic stem (ES) cells are derived from blastocyst-stage embryos. Their unique properties of self-renewal and pluripotency make them an attractive tool for basic research and a potential cell resource for therapy. ES cells of mouse and human have been successfully generated and applied in a wide range of research. However, no genuine ES cell lines have been obtained from rat to date. In this study, we identified pluripotent cells in early rat embryos using specific antibodies against markers of pluripotent stem cells. Subsequently, by modifying the culture medium for rat blastocysts, we derived pluripotent rat ES-like cell lines, which expressed pluripotency markers and formed embryoid bodies (EBs) *in vitro*. Importantly, these rat ES-like cells were able to produce teratomas. Both EBs and teratomas contained tissues from all three embryonic germ layers. In addition, from the rat ES-like cells, we derived a rat primitive endoderm (PrE) cell line. Furthermore, we conducted transcriptional profiling of the rat ES-like cells and identified the unique molecular signature of the rat pluripotent stem cells. Our analysis demonstrates that multiple signaling pathways, including the BMP, Activin and mTOR pathways, may be involved in keeping the rat ES-like cells in an undifferentiated state. The cell lines and information obtained in this study will accelerate our understanding of the molecular regulation underlying pluripotency and guide us in the appropriate manipulation of ES cells from a particular species.

***Keywords*:** embryonic stem cells, blastocysts, primitive endoderm, teratomas
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Introduction

Pluripotent mouse embryonic stem (ES) cells are derived from the inner cell mass (ICM) of blastocyst-stage embryos [1] and they have been proven capable of indefinite self-renewal and of retention of their pluripotent potential to form all cell types *in vitro* [2-4]. In addition, they can be genetically engineered and can contribute to all three embryonic germ layers and to the germ line in chimeras when injected into host blastocysts, offering a unique opportunity to produce live mice with a desired mutation in every cell [5]. Therefore, mouse ES cells have served as a valuable model and an important tool in studying mammalian development. More recently, human ES cell lines were successfully generated, making ES cells also an attractive source for cell therapy [6].

Apart from ES cells from mouse and human, scientists have made considerable efforts to generate pluripotent ES cell lines from different vertebrate species, in particular, from rat [7-9], an important model for biomedical research and for study of human diseases. However, the attempts to establish genuine ES cell lines from rat have been unsuccessful to date. Several reports describe "rat ES-like cells", which grew like ES cells in culture [8-12]. However, the developmental capacity of these cells was not well characterized *in vivo*. It is worth noting that in the published reports, rat ES-like cells were derived

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under similar conditions to those used for generation of mouse ES cells. In fact, differences in intrinsic biology between mouse and rat have been observed [13]. Therefore, understanding the molecular mechanisms of pluripotency in rat embryonic pluripotent cells is an essential step towards successful derivation and application of rat ES cells. Nevertheless, there has been a lack of transcriptional profiling of rat pluripotent cells and we have accumulated little information about optimal conditions for derivation and maintenance of rat ES cells in culture.

In this study, we have successfully derived and characterized rat ES-like cell lines, which not only express pluripotency markers and form well-organized embryoid bodies (EBs), but also produce various cell types of the three embryonic germ layers in EBs and teratomas. In addition, we generated a rat primitive endoderm (PrE) cell line from the rat ES-like cells. To identify the molecules uniquely expressed in the undifferentiated rat ES-like cells, we performed transcriptional profiling of the rat ES-like cells and PrE cells. The molecular signature of the undifferentiated rat ES-like cells has been carefully analyzed. The cell lines and information obtained in this study will accelerate our understanding of molecular mechanisms of pluripotency and guide us in the proper manipulation of ES cells from a particular species.

Results

Localization of pluripotent cells in rat early embryos

Mouse ES cells have been successfully derived from the ICM of blastocysts expressing Oct4, Nanog and Sox2. However, the localization of pluripotent cells expressing these marker genes in rat early embryos is poorly characterized. To isolate rat ES cells, we began by examining the localization of Oct4-expressing cells in rat embryos at the blastocyst stage (4.5 day post coitus, dpc). Immunofluorescent imaging revealed that Oct4-staining was restricted in the ICM, but not in the

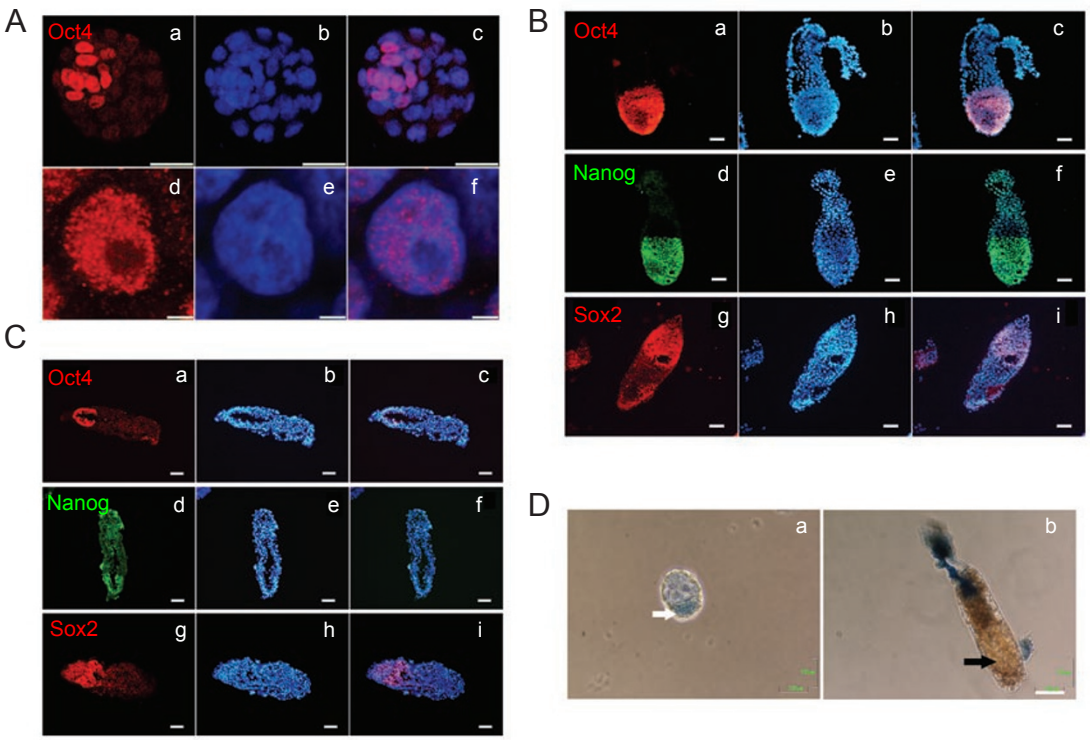


Figure 1 Localization of pluripotent cells in early rat embryos. **(A)** The rat Oct4 protein (red) is detected in the ICM of 4.5 dpc rat blastocyst (a). Punctate staining of Oct4 is observed in all nuclei (absent in nucleoli) (d). DAPI staining (blue) highlighting the nuclei is shown in panels b and e. The composite images are shown in panels c and f. The scale bars in a-c are 25 μm and in d-f are 2.5 μm. **(B)** Expression of pluripotency markers, Oct4 (red, a), Nanog (green, d) and Sox2 (red, g), is detected in the epiblast of rat 7.5 dpc embryo at egg cylinder stage. Corresponding DAPI staining is shown in panels b, e and h. The composite images are shown in panels c, f and i. The scale bars are 50 μm. **(C)** Expression of Oct4 (red, a), Nanog (green, d) and Sox2 (red, g) is examined in the mouse 6.0 dpc embryo at egg cylinder stage. Corresponding DAPI staining is shown in panels b, e and h. The composite images are shown in panels c, f and i. The scale bars are 50 μm. **(D)** AKP staining is positive in the ICM of the rat 4.5 dpc embryo at the blastocyst stage (a, white arrow). No signal is observed in the epiblast of the rat 7.5 dpc embryo at the egg cylinder stage (b, black arrow). The scale bars are 100 μm.

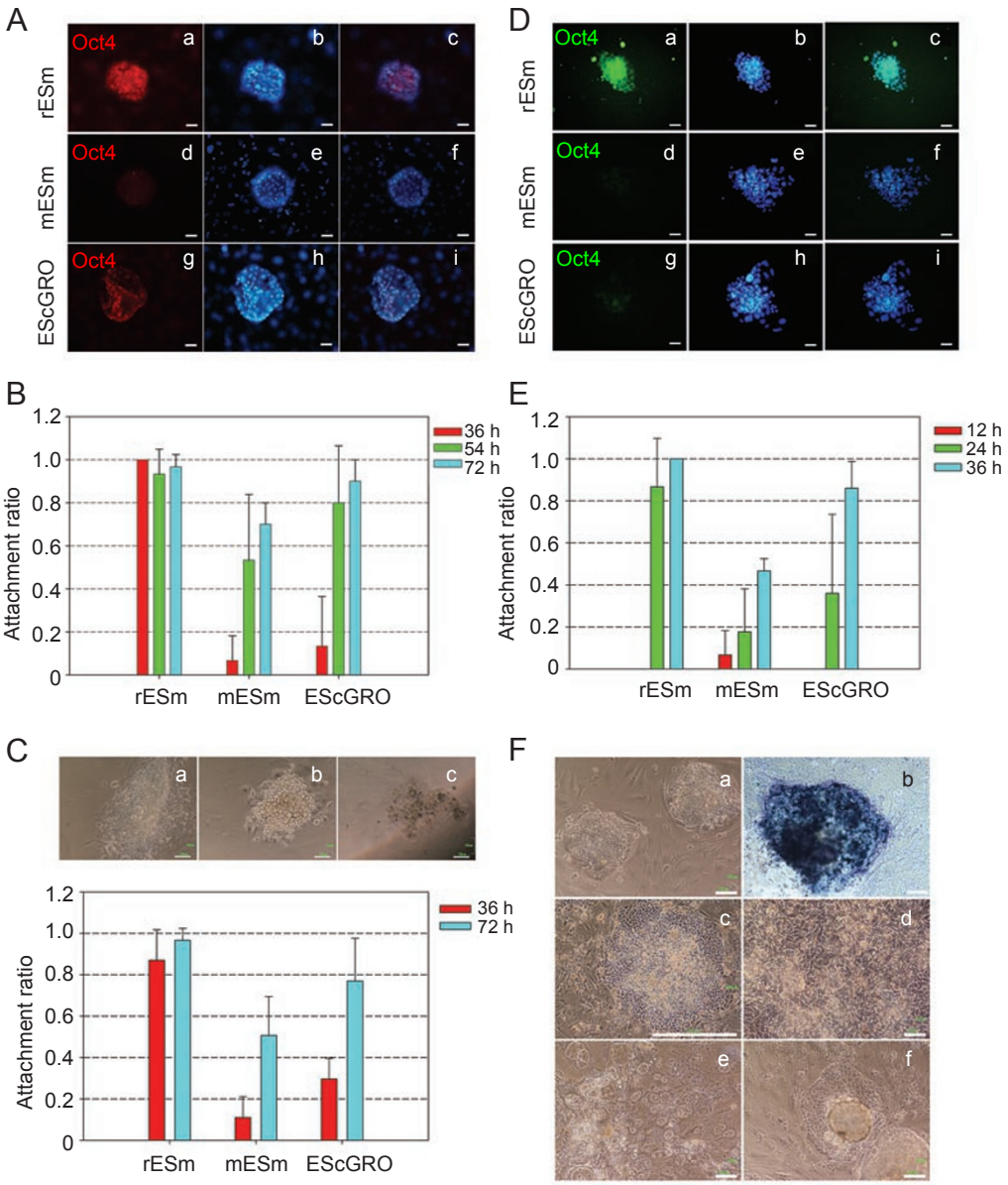


Figure 2 Identification of the optimal medium for derivation of the rat ES-like cells. **(A)** Whole rat blastocysts were cultured in rat ES cell medium (a-c), mouse ES cell medium (d-f) and EScGRO (g-i). Oct4 (red) expression was detected by immunostaining after 72 h in primary culture. Corresponding DAPI staining is shown in panels b, e and h. The composite images are shown in panels c, f and i. The scale bars are 50 μm. **(B)** While rat blastocysts were cultured under feeder-dependent conditions, their attachment ratio in different culture media was quantified. The columns display the attachment ratio. The error bar displays standard deviation from three independent experiments. **(C)** When rat blastocysts were cultured under feeder-free conditions, images of their morphology were captured in the rat ES cell medium (a), the mouse ES cell medium (b) and EScGRO (c). The attachment ratio of rat blastocysts in the different culture media was quantified (lower panel). The columns display the attachment ratio. The error bar displays standard deviation from three independent experiments. The scale bars are 100 μm. **(D)** Zona pellucida free rat blastocysts were cultured in the rat ES cell medium (a-c), mouse ES cell medium (d-f) and EScGRO (g-i). Oct4 (green) expression was detected by immunostaining after 12 h in primary culture. Corresponding DAPI staining is shown in panels b, e and h. The composite images are shown in panels c, f and i. The scale bars are 50 μm. **(E)** When zona pellucida free rat blastocysts were cultured under feeder-dependent conditions, their attachment ratio in the different culture media was quantified. The columns display the attachment ratio. The error bar displays standard deviation from three independent experiments. **(F)** The mechanically passaged rat ES-like cells maintain typical morphology of an ES cell colony (a) and are positive for AKP staining (b). The enzymatically passaged rat ES-like cells maintain a similar morphology (c-d). The differentiated trophoblast giant cells (e) and endoderm-like cells (f) are observed in the periphery of the partially differentiated rat ES-like cell colonies. The scale bar in c is 1 000 μm and the rest are 100 μm.

peripheral trophectodermal cells (Figure 1A). The staining of Oct4 in the nuclei of the ICM cells was punctate, being absent from the nucleoli. Furthermore, we checked the localization of pluripotent cells in rat embryos at 7.5 dpc. Mouse embryos at 6.0 dpc were also examined as a control. As shown in Figure 1B, the positive signals for Oct4, Nanog and Sox2 were all localized in the epiblast of rat embryos. In contrast, extra-embryonic tissues were negative for all three antibodies. Moreover, staining for endoderm markers, Gata4 and Hnf4α, was positive in rat extra-embryonic endoderm cells, but not in the epiblast cells (Supplemental imformation Figure S1). Similar to rat embryos, Oct4-, Nanog- and Sox2-positive cells were detected in the epiblast of mouse embryos (Figure 1C), consistent with previous reports [14, 15]. These observations indicate that embryonic pluripotent cells are localized in the ICM and in the epiblast of early rat embryos. To further confirm the localization of the pluripotent cells, expression of another pluripotency marker, alkaline phosphatase (AKP), was examined in the ICM of blastocyst and the epiblast of the egg cylinder of rat embryos (Figure 1D). Our results show that AKP staining was positive in the ICM cells, but became negative in the epiblast of the egg cylinder, consistent with the AKP staining pattern seen in mouse embryos [16, 17].

Optimization of culture medium for derivation of rat ES-like cells

Knowing that the expression pattern of pluripotency markers in early mouse and rat embryos is similar (Figure 1), we tried to derive rat ES cell lines utilizing a medium similar to that used for mouse ES cell derivation. However, after rat blastocysts were plated onto mouse embryonic fibroblast (MEF) cells, the ICM cells did not grow well in either mouse ES cell medium or in the component defined medium (EScGRO, Millipore). Further study showed that rat blastocysts cultured in the mouse ES cell medium readily differentiated into trophoblast giant cells, while embryos cultured in the EScGRO markedly differentiated into endoderm-like cells (data not shown). The phenomenon suggested to us that the mouse ES cell medium might contain factors inhibitory to primitive endoderm commitment and that the EScGRO might contain components inhibitory to trophoblast giant cell differentiation. We thus hypothesized that the combination of the two media might promote ICM cell proliferation in an undifferentiated state. To test the hypothesis, we mixed the mouse ES cell medium with EScGRO at a 1:1 ratio and included in the mixture 2 μM GSK-3 selective inhibitor (BIO), which had been demonstrated to help maintain both mouse and human ES cells in an undifferentiated state in culture [18, 19]. We named the mixture "rat ES cell medium". When rat embryos cultured for 5 days were stained with antibody against Oct4, we observed significantly more Oct4-positive cells using the rat ES cell medium as compared to cultures using either the mouse ES cell medium or EScGRO (Figure 2A). To determine why more Oct4-postive cells were obtained with the rat ES cell medium, we compared the attachment ratio of rat blastocysts in the three culture media. After whole blastocysts were plated in the rat ES cell medium, almost 100% of blastocysts attached to MEF cells within 36 h. However, less than 20% were found to attach either in the mouse ES cell medium or EScGRO. Even after 72 h, only approximately 70% and 90% of blastocysts attached in the mouse ES cell medium and EScGRO, respectively (Figure 2B). When the same experiments were repeated in matrigel-coated culture plates, obvious improvement of the embryo attachment ratio was also obtained with the rat ES cell medium, particularly within 36 h (Figure 2C). We believe that acceleration of attachment of blastocysts is the key factor contributing to the greater number of Oct4-positive cells observed in the rat ES cell medium. To further confirm this assumption and exclude the influence of zona pellucida on hatching, we treated the whole rat blastocysts with Tyrode's solution to destroy the zona pellucida and repeated the embryo attachment experiment. Again, more Oct4-positive cells

Table 1 Derivation Efficiency of rat ES-like cell lines

Experiment	No. of blastocysts	Cell line	Name	Teratoma (three germ layer)	Efficiency
I	11	1	rES1222	Yes/intramuscular injection	9%
II	8	1	rES0107	Yes/subretinal injection	12.5%
			rES1121	Yes/subretinal injection	
III	8	3	rES1122	N.D.	37.5%
			rES0113	Yes/subretinal injection	
				Yes/subretinal injection (p6)	
IV	4	1	rES3151	Yes/intramuscular injection (p16)	25%
Total	31	6			19%

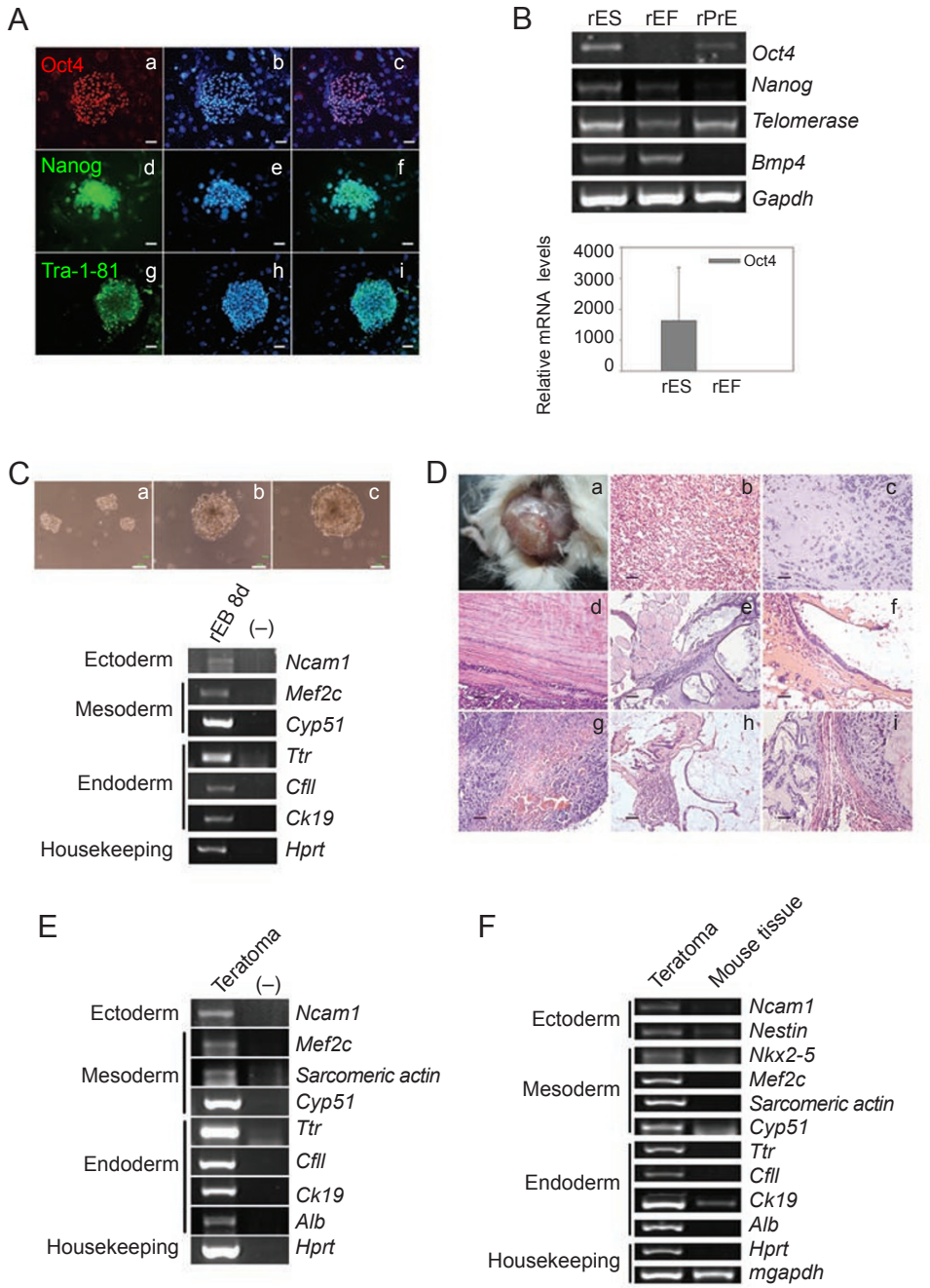


Figure 3 Characterization of rat ES-like cells *in vitro* and *in vivo*. **(A)** The cultured rat ES-like cells at passage 7 are Oct4- (red, a), Nanog- (green, d) and Tra-1-81- (green, g) positive. Corresponding DAPI staining is shown in panels b, e and h. The composite images are shown in panels c, f and i. The scale bars are 50 μm. **(B)** Expression of pluripotency marker genes in rat ES-like cells was examined by the RT-PCR analysis at passage 12 and compared with expression levels in rEF cells and rat PrE cells. Quantitative real time PCR analysis for expression of the rat Oct4 level was done in different passages of rat ES-like cell lines 3151 and 1222. The error bar displays standard deviation from three independent experiments. **(C)** The rat ES-like cells form cystic EBs in serum containing medium (a). The PrE cells appear in the periphery of EBs from day 7 (b) and EBs grow significantly at day 9 (c). The scale bars are 100 μm. The RT-PCR analysis of the three germ layer markers confirms the existence of various tissues in the EBs formed from rat ES-like cells. **(D)** Teratomas were harvested 4 months after intramuscular injection of the rat ES-like cells into SCID mice (a). Various tissues such as myelinated nerve fiber (b); cartilage (c); smooth muscle (d); fat (e); columnar epithelium of lung (f); blood vessel (g); goblet cells (h) and skeletal muscle (i) were detected by histochemical staining. The scale bars are 50 μm. **(E)** RT-PCR analysis of the three germ layer markers confirms the existence of various tissues in the teratomas derived from the rat ES-like cells. **(F)** RT-PCR analysis of the three germ layer markers confirms the specificity of the primers used for the analysis of rat genes in teratomas.

and a significant improvement of the embryo attachment ratio were obtained with the rat ES cell medium, although embryos attached to the matrigel much sooner in the absence of the zona pellucida than in its presence for all three media (Figure 2D-2E). These observations favor our conclusion that the rat ES cell medium has an ability to enhance embryo attachment during rat ES cell derivation. With the enhanced blastocyst attachment in the rat ES cell medium, we were able to mechanically passage the rat ES-like cells in the early five passages. Undifferentiated rat ES-like cells with a high nucleus to cytoplasm ratio formed compacted colonies. The periphery of the rat ES-like colony was distinct from MEF cells and the rat ES-like cells were AKP-positive (Figure 2F, a, b). When passaged with trypsin, approximately 50-70% of cells displayed undifferentiated cell morphology, which appeared homogenous with a high nucleus to cytoplasm ratio (Figure 2F, c, d). The rest of the cells, especially those in the periphery of the colonies, exhibited differentiated morphology. Two types of differentiated morphology were found, one resembling trophoblast giant cells (Figure 2F, e), and the other resembling endoderm cells (Figure 2F, f). The trophoblast giant cells gradually vanished during continuous culture, but the endoderm-like cells remained in the partially differentiated colonies. At present, six rat ES-like cell lines from thirty-one rat blastocysts have been generated (Table 1) and one of them (line 3151) has been continuously passaged for four months (more than twenty passages). Moreover, the cells can be frozen and thawed successfully.

Characterization of rat ES-like cell lines

One of the criteria for pluripotent stem cells is their expression of a group of pluripotency marker genes. As shown in Figure 3A, the nuclear localization of Oct4, Nanog and membrane localization of Tra-1-81 were observed in the undifferentiated rat ES-like cells (two independently derived rat ES-like cell lines, line 1222 and line 3151). In contrast, MEF cells were negative for these antibodies. Due to the limited availability of antibodies reactive to rat antigens, we also collected RNA samples from the rat ES-like cells, rat embryonic fibroblast (REF) cells and rat primitive endoderm (PrE, see following section) cells to determine expression levels of several marker genes (Figure 3B). RT-PCR analysis showed that *Oct4* and *Nanog* were highly expressed in the undifferentiated rat ES-like cells, whereas their expression in the rat PrE and REF cells was weak. Quantitative real time PCR (qPCR) analysis further confirmed distinctly higher expression of *Oct4* in rat ES-like cells as compared to REF cells (Figure 3B, bottom panel). Furthermore, the expression level of the rat *Tert* gene was highest in the rat ES-like cells. Modest and weak expression of the rat *Tert* gene was detected in the rat PrE and REF cells, respectively. *Bmp4* was reported as an important cytokine mediating the pluripotent state and mesoderm differentiation in mouse ES cells [20, 21]. We found high expression of *Bmp4* in the rat ES-like cells as well as in the REF cells.

The second hallmark of ES cells is their potential to form all cell types of the three embryonic germ layers both *in vitro* and *in vivo*. The *in vitro* differentiation potential of mouse and human ES cells has often been tested using the EB formation assay. Our rat ES-like cells formed EBs as mouse and human ES cells usually do. After culture for 6-8 days, two distinct layers were observed in the EBs, the interior layer in the center and the outer layer on the periphery of the EBs (Figure 3C). A similar phenomenon was always observed in mouse EBs, in which the outer layer primarily contains PrE cells [22]. RT-PCR analysis confirmed the presence of markers for the three germ layers in our EBs, such as *Ncam1, Mef2c, Cyp51, Ttr, CfII* and *Ck19* (Figure 3C, bottom panel). This observation demonstrates that our rat ES-like cells have the ability to differentiate normally *in vitro*. To test their differentiation ability *in vivo*, a teratoma formation assay was performed by intramuscular methods for the rat ES cell lines 1222 and 3151 in SCID mice. Four months after injection, teratomas were collected from recipient mice (Figure 3D, a). In the teratoma sections, we observed typical myelinated nerve fiber (ectoderm), cartilage, smooth muscle, skeletal muscle, fat (mesoderm) and columnar epithelium of lung (endoderm) (Figure 3D, b-i). In addition, the expression of marker genes for all three germ layers was readily detected in the RNA samples extracted from the rat ES-like cell-derived teratomas, but not in the RNA samples which were not reversely transcribed, revealing that the RT-PCR signals originated from the RNA in our samples, but not from potentially contaminating genomic DNA (Figure 3E). To ensure the specificity of the PCR primers used for the rat samples, we assessed the gene expression levels in the rat ES cell-derived teratomas and mouse tissues, using the same primers. As shown in Figure 3F, the primers exclusively recognized the rat RNA extracted from teratomas, but did not react to the RNA extracted from mouse tissues. These data provide strong evidence that our rat ES-like cells have pluripotent differentiation potential. In addition, three other rat ES-like cell lines also generated teratomas using subretinal injection methods (data not shown).

Rat PrE cell line derived from rat ES-like cells

As mentioned above, there were PrE-like cells in the periphery of the partially differentiated colonies (Figure

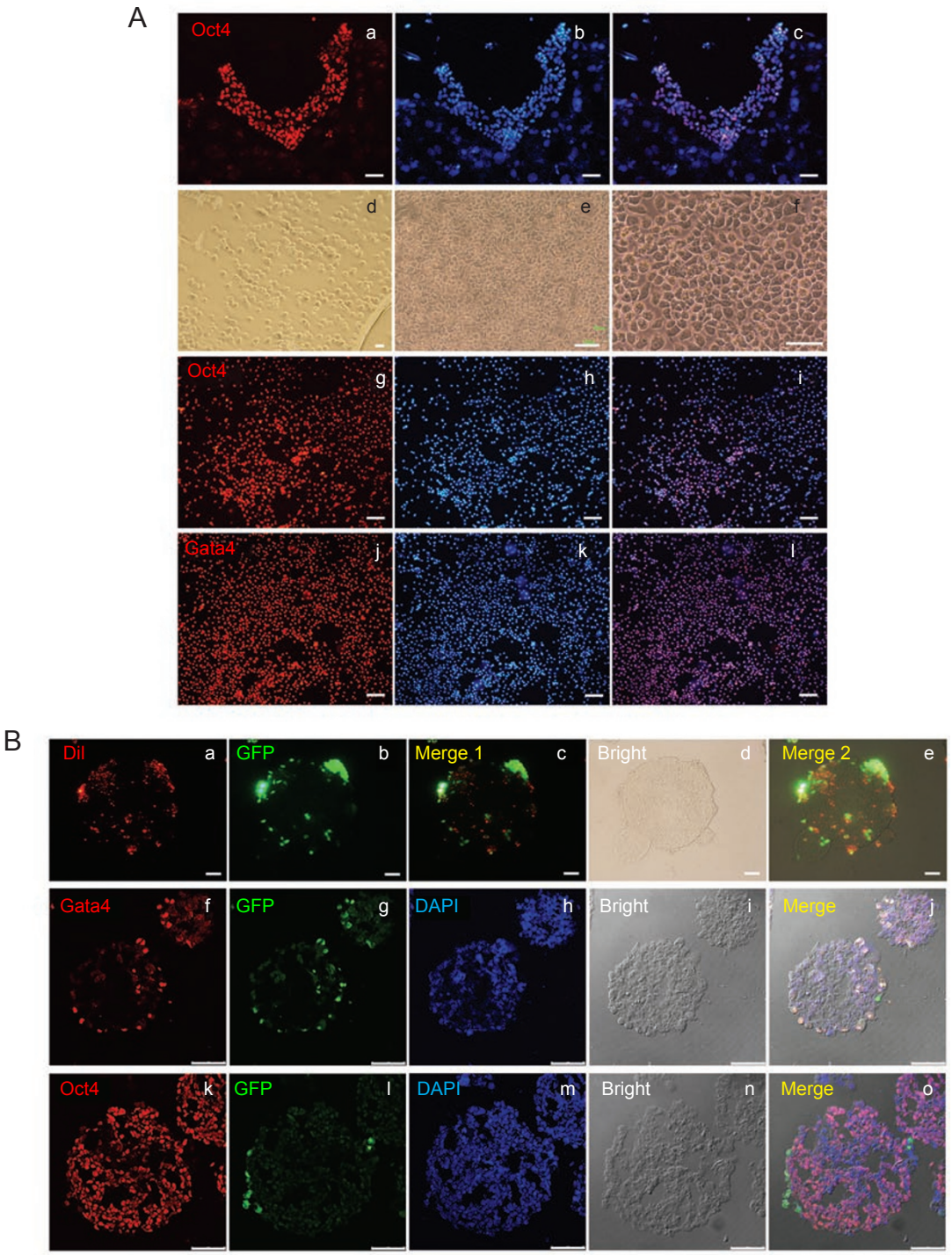


Figure 4 Characterization of rat PrE cells differentiated from rat ES-like cells. **(A)** The endoderm-like cells exist in the periphery of the partially differentiated rat ES-like colony. After the undifferentiated rat ES-like cells in the center of the colony were mechanically removed, the remaining endoderm-like cells located in the periphery of the colonies were stained by Oct4-antibody (red, a). When these differentiated cells were transferred to the gelatin-coated culture dish and passaged continuously (d-f), they were seen to be positive for Oct4 (red, g) and Gata4 (red, j) by immunostaining at passage 5. Corresponding DAPI staining is shown in panels b, h and k. The composite images are shown in panels c, i and l. The scale bars in a-c are 50 μm and in d-l are 100 μm. **(B)** The GFP and DiI double-labeled rat PrE cells at passage 7 are seen to migrate to the periphery of chimeric EB when mixed with mouse ES cells (a-e). These GFP labeled rat PrE cells express Gata4 (red, f) and Oct4 (red, k) as detected by immunostaining. The scale bars in a-e are 50 μm and in f-o are 75 μm.

2F, f). Interestingly, these cells expressed Oct4 (Figure 4A, a). To characterize the PrE-like cells in detail, we derived the rat PrE cell line by mechanically transferring the PrE-like cells onto gelatin-coated cell culture dishes and culturing the cells in the mouse ES cell medium in the absence of leukemia inhibitory factor (LIF), a factor routinely used in mouse ES cell culture. We were able to passage these cells *in vitro* for more than 10 passages using trypsin (Figure 4A, d-f). The cells stably maintained PrE cell morphology. Immunostaining also showed that Oct4 could be detected in almost all the cells examined (Figure 4A, g), as could the endoderm marker Gata4 (Figure 4A, j). To test whether the rat PrE cells behaved like mouse PrE cells, we conducted the chimeric EB formation assay, which has been shown to be effective in determining whether the tested cells are of PrE origin. The assay is based upon the phenomenon that PrE cells exclusively migrate to the surface of EBs when mixed with undifferentiated mouse ES cells in suspension culture [23]. Thus, we mixed rat PrE cells (double labeled with GFP and DiI dye) with unlabeled mouse ES cells. As expected, almost all the GFP and DiI double labeled rat PrE cells became positioned in the periphery of the chimeric EBs (Figure 4B, a-e), demonstrating that rat PrE cells have the ability to migrate and integrate into the outer layer of EBs, as would mouse PrE cells. However, when we mixted rat ES-like cells stained by DiI with the mouse ES cells, we could see the even distribution of the rat ES-like cells throughout the chimeric EBs (data not shown). Furthermore, we examined the spatial expression pattern of Oct4 and Gata4 in the chimeric EBs. Clearly, Gata4-positive cells were co-localized with GFP-labeled PrE cells in the periphery of the EBs (Figure 4B, f-j). However, Oct4 was found in both interior cells and the outer layer of the EBs (Figure 4B, k-o), consistent with our observation that Oct4 is expressed in both the undifferentiated rat ES-like cells and the PrE cells. These data provide the functional evidence for the identity of our derived PrE cell line.

Genome-wide transcriptional profiling of rat ES-like cells

To identify specifically expressed genes in the undifferentiated rat ES-like cells, we performed transcriptional profiling of these cells using Affymetrix rat expression array 230 2.0. The Chip included 31 099 probe sets, in which 11 993 probe sets were related to ESTs. The rat PrE cell line was chosen as the reference, since these cells were observed in the culture of rat ES-like cells. 629 transcripts were selected as genes enriched specifically in the undifferentiated rat ES-like cells based upon up-regulation of their expression level by more than 2-fold in the rat ES-like cells as compared to the level in the PrE cells and based upon their detection at significant levels (Affymetrix "P" call). Among them, 418 transcripts had the gene symbol and the other 211 transcripts were related to ESTs (Supplementary information, Table S1). The transcripts with annotation which were up-regulated by more than 10-fold in the rat ES-like cells are shown in Figure 5A.

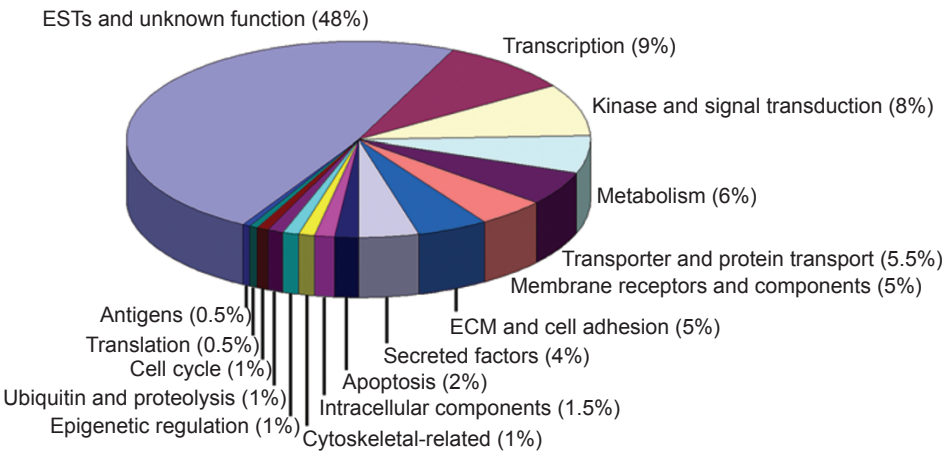
All the rat ES-like cell-enriched genes were classified according to the functional annotation on NetAffx analysis center (https://www.affymetrix.com/analysis/netaffx/index.affx) (Figure 5B and Supplementary information, Table S1). Among the 629 transcripts, 303 (48%) were related to transcripts of unknown function and ESTs. Surprisingly, for the other 326 transcripts with functional annotation, the activity in regard to maintaining ES cell pluripotency was not found for most of them in the PubMed database. Nevertheless, there were molecules which are known to be highly expressed in undifferentiated human or mouse ES cells, such as *Thy1*, *Fbxo15*, *Gbx2*, *Dnmt3a* and *Dnmt3b,* which were also enriched in the rat ES-like cells. In fact, many transcription factors were enriched (9%). *Klf2*, encoding a Krüppel-like factor, was highly expressed in the rat ES-like cells. Core Klf circuitry including Klf2, Klf4 and Klf5 has been reported to have a synergistic effect on self-renewal of mouse ES cells and to regulate key pluripotency genes, including *Nanog* [24]. The high expression level of *Klf2* implies a role for the Klf molecule in rat ES-like cells. Moreover, the other two groups of molecules, secreted factors and membrane receptors/components, made up 4% and 5% of the collection, respectively. For instance, *Igf1* and *Igf2* were enriched, together with *Igf2r*. In addition, *Fgfr1* and *Fgfr2* were all highly expressed in the undifferentiated rat ES-like cells, suggesting that these pathways could be activated in an autocrine or paracrine manner in rat ES-like cells (Figure 5C). Exogenous FGF2 is required to maintain human ES cells in the undifferentiated state [6] and IGF1 has a positive effect on human ES cell growth [25]. The expression of these ligands and receptors suggests critical roles for the endogenous IGF and FGF signaling pathways in the growth of rat ES-like cells. Further analysis of these enriched genes will probably reveal new molecular networks active in rat ES-like cells.

Signal transduction is essential for diverse biological processes. The FGF signaling pathway is known to play an essential role in maintenance of the undifferentiated state in human ES cells [6, 26], and self-renewal of mouse ES cells is known to be dependent upon the JAK/STAT signaling pathway [27]. To determine the signaling pathways active in rat ES-like cells, we analyzed the KEGG pathways of the selected 629 rat transcripts

A

Gene Title	Gene Symbol	Unigene	GenBank
procollagen, type I, alpha 2	Col1a2	Rn.107239	NM_053356.1
inhibin beta-B	Inhbb	Rn.35074	XM_344130.3
chemokine (C-C motif) ligand 2	Ccl2	Rn.4772	NM_031530.1
procollagen, type V, alpha 2	Col5a2	Rn.2875	XM_343564.3
twist homolog 2 (Drosophila)	Twist2	Rn.16279	NM_021691.2
Procollagen, type V, alpha 1	Col5a1	Rn.117	NM_134452.1
chondroitin sulfate proteoglycan 2	Cspg2	Rn.35666	XM_215451.4
glypican 3	Gpc3	Rn.9717	NM_012774.1
cadherin 11	Cdh11	Rn.8900	XM_001059464.1
Iroquois related homeobox 1 (Drosophila)	Irx1_predicted	Rn.46018	XM_225101.4
chemokine (C-C motif) ligand 7	Ccl7	Rn.26815	NM_001007612.1
procollagen, type 1, alpha 1	Col1a1	Rn.2953	NM_053304.1
insulin-like growth factor 2	Igf2	Rn.118681	NM_031511.1
transcription factor AP-2, gamma	Tcfap2c	Rn.16142	NM_201420.2
EF hand domain family A1	Efha1	Rn.64644	NM_134396.2
ceruloplasmin	Cp	Rn.32777	NM_012532.1
eukaryotic translation elongation factor 2	Eef2	Rn.55145	NM_017245.2
neurotrophin 3	Ntf3	Rn.9715	NM_031073.2
actin, gamma, cytoplasmic 1	Actg1	Rn.101464	NM_001127449.1
Tenascin C	Tnc	Rn.12723	
dihydropyrimidinase-like 3	Dpysl3	Rn.93365	NM_012934.1
matrix metalloproteinase 16	Mmp16	Rn.208361	NM_080776.1
cytokine receptor-like factor 1 (predicted)	Crlf1_predicted	Rn.38637	NM_001106074.1
inhibin beta-A	Inhba	Rn.9874	NM_017128.1
procollagen, type VIII, alpha 1 (predicted)	Col8a1_predicted	Rn.53843	NM_001107100.1
paired related homeobox 2 (predicted)	Prrx2_predicted	Rn.93004	NM_001105739.1
placenta-specific 1	Plac1	Rn.24205	NM_001024894.1
glycine decarboxylase (predicted)	Gldc_predicted	Rn.17101	NM_001107583.1

B



C

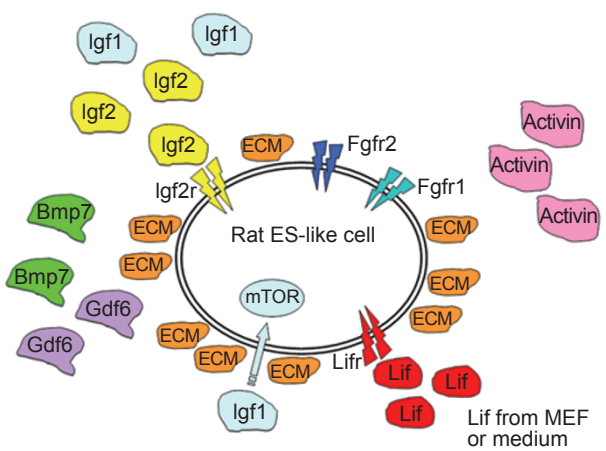


Figure 5 Functional annotation of rat ES-like cells enriched genes. **(A)** The enriched genes, which are expressed more than 10-fold in the rat ES-like cells. **(B)** A total of 629 of the rat ES-like cells enriched transcripts are divided into 16 groups according to their functional annotation. The full list is available in the Supplementary information, Table S1. **(C)** Multiple signaling pathways present in rat ES-like cells.

by DAVID tools (Supplementary information, Table S2 and Figure 5C). Overall, we found that the TGFβ/BMP pathway is enriched in rat ES-like cells containing components such as *Dcn, Gdf6, Bmp7, Inhbb* and *Inhba* (*Activin*). This pathway has been shown to be involved in pluripotency of both mouse and human ES cells, in which Bmp is important for mouse ES cells while Activin is essential for human ES cells [20, 28, 29]. The existence of both *Bmp* and *Activin* transcripts suggests a unique signal transduction mechanism in the maintenance of rat ES-like cells. Furthermore, several transcripts of the mTOR signaling pathway (*Igf1*, *Figf*, *Ddit4* and *Fyb*) were enriched in the undifferentiated rat ES-like cells. Although the mechanism of the mTOR signaling pathway in the rat ES cells remains obscure, it is essential for proliferation of mouse ES cells and early embryos [30]. On the other hand, our analysis did not detect enrichment of the JAK/STAT signaling pathway in rat ES-like cells. However, we found that *Lifr,* but not *Lif,* was termed "present" in the rat ES-like cells. It is possible that Lif produced by the feeder layer or provided by the culture media activates Lifr present in rat ES-like cells to help maintain them in an undifferentiated state. The finding confirmed the rationality of our inclusion of LIF in the culture medium for derivation of rat ES-like cells. Another important pathway that influences the proliferation of both mouse and human ES cells is the Wnt signaling pathway [31, 32]. Unexpectedly, the Wnt signaling pathway was not found to be enriched in the rat ES-like cells. Wnt4 was the only enriched component and it has been reported to be not essential for supporting growth of ES cells [33].

In addition to the signaling pathways, another interesting finding from our analysis was that the extracellular matrix (ECM)-receptor interaction was active in rat ES-like cells. For example, *Sdc3, Fndc1, Spp1, Col3a1, Col5a3, Col5a2, Col5a1, Col1a1* and *Col1a2* were all highly expressed in these cells. The fact that human ES cells can proliferate on matrigel without feeders reflects the important role of ECM in maintaining ES cells [34]. The function of these enriched ECM transcripts is not clear, but their high level of expression supports our early observation that embryo attachment was a critical factor for derivation of the rat ES-like cells.

Discussion

Here, we report high efficiency generation of ES-like cells from rat blastocysts, which express pluripotency markers Oct4, Nanog, Tra-1-81 and AKP and produce a variety of cell types originating from all three embryonic germ layers in both EBs and teratomas. The result is significant in that this is the first report that rat ES-like cell lines are able to contribute to the three embryonic germ layers *in vivo*, an essential criterion in demonstrating the true pluripotent character of ES cell lines. Our result marks an important step forward in the generation of genuine rat ES cells with the capacity to produce chimeras and germ line transmissions.

We believe that one of the critical factors that allowed us to successfully produce pluripotent rat ES-like cells in this study was our identification of an optimal rat blastocyst culture medium, which promoted the attachment of cultured blastocysts to the substrate and enhanced the number of cells expressing Oct4 in the embryos, although its exact components are not yet defined. Meanwhile, we found that the rat ES cell medium did not increase the hatching of rat blastocysts (data not shown). Many researchers have experienced considerably more differentiation of cultured rat blastocysts compared to mouse blastocysts. This could be due to the distinctive developmental potential of the early pluripotent cells between these two closely related rodents. In fact, Nichols and Buehr demonstrated that rat epiblasts produced extra-embryonic endoderm cells in culture, whereas mouse epiblasts did not [13]. Therefore, it is reasonable to expect that the longer the embryos are suspended in medium, the more differentiated cells would be produced from the epiblast. In contrast, when rat embryos attach to the substrate quickly and propagate soon after the epiblasts grow out, the undifferentiated pluripotent cells can be isolated before a massive differentiation occurs. It appears that the first 36 h after whole embryos are plated onto substrate is the critical period for their further development. Importantly, the early attached embryos in the rat ES cell medium express the Oct4 protein at a substantially higher level than the later attached embryos in either the mouse ES cell medium or the EScGRO. This finding is consistent with the notion proposed by Buehr *et al.* [8] that the continuous expression of Oct4 through the initial stages of derivation may be necessary if pluripotent cell lines are to be established.

Previously, several attempts at derivation of rat ES-like cell lines have been reported. Vassilieva *et al.* [12] described the establishment of rat ES-like cell lines, in which the *Oct4* mRNA level was found to be lower than that of mouse ES cells. Although the cells displayed typical growth of highly compacted cell clusters, the capacity of these cells to differentiate was not presented. Later, long-term culture and differentiation of rat ES-like cells into neuronal, glial, endothelial, and hepatic lineages *in vitro* was reported by Ruhnke *et al*. [11]. However, the cells were not characterized for their expression of Oct4. Recently, Demers *et al.* [9] described efficient deriva-

tion of rat ES-like cells expressing pluripotency markers. Interestingly, their ES-like cells, when injected into blastocysts, contributed to extra-embryonic tissues, but not to embryonic germ layers. Collectively, these published results have not shown that the cognate ES-like cells possess pluripotent differentiation potential *in vivo*. In this study, we generated six rat ES-like cell lines. Two of the lines have been extensively characterized, except for their capacity to contribute to chimeras and produce germ line transmissions, a circumstance which is now under examination in our laboratory. The establishment of such cell lines provides an opportunity for further investigation of molecular regulation in rat pluripotent cells and their potential utilization in targeted genetic modification, which could widen the application of rat disease models in biomedical research. In addition to rat ES-like cells, we also generated rat PrE cells, which can be reproducibly isolated from the differentiated rat ES-like cells. Microarray analysis showed that they express at high levels molecular markers for PrE cells, such as *Gata6, Gata4, Lamb1, Sox7, Sox17* and *Foxa2*. Most convincingly, these cells display functional properties of PrE cells. Surprisingly, these PrE cells express Oct4, reminiscent of a previous report of transiently high expression of Oct4 in the PrE cells of mouse early embryos [35]. Currently, the function of Oct4 in rat PrE cells remains unclear. However, the established PrE cell line enables us to further investigate the unique molecular network operating in early rat embryos.

The failure in the establishment of true rat ES cell lines is primarily due to the lack of understanding of molecular regulation in rat pluripotent cells. Our transcriptional profiling and analysis of rat ES-like cells not only provide a list of the transcripts enriched in these undifferentiated cells but also suggest that multiple signaling pathways, such as those of TGFβ, mTOR, IGF, FGF and ECM-receptor interaction, might be involved in maintaining self-renewal and pluripotency of the undifferentiated rat ES-like cells. In addition, LIF is perhaps a very important factor for rat ES cells. Our results indicate that rat ES-like cells share some characteristics with either mouse or human ES cells at the molecular level, while they also have their own unique transcriptional landscape. In the 629 enriched transcripts of the rat ES-like cells, few of them have been reported to be involved in maintaining pluripotency of ES cells. However, it should be noted that several core pluripotency genes such as *Oct4* and *Nanog* were not included in the probes of the rat expression array 230 2.0 Chip, although our immunostaining experiments showed their specific expression in rat ES-like cells. The apparent lack of enrichment of previously known pluripotency-related genes could have resulted from the shortage of rat genome information and lack of annotation for many transcripts. It is also highly possible that rat ES cells have their own distinctive regulatory mechanisms for maintaining self-renewal and pluripotency. Taken together, our data provide a list of uniquely expressed genes and important pathways in rat ES-like cells for further in-depth investigation. Identification of these molecular features will help us to optimize conditions for derivation and culture of true rat ES cell lines. In addition, we obtained microarray data from REF cells and compared them to those collected from rat ES-like cells. Such a comparison led to the enrichment of many endoderm marker genes for the rat ES-like cells, including *Foxa2*, *Gata6*, *Gata4*, *Sox7*, *Sox17*, *Ihh*, *Hnf4α*, *Krt1-19* and *Krt1-18*. Further analysis indicated that these genes are highly expressed in both our rat ES-like cells and rat PrE cells. To obtain the transcripts uniquely expressed in the undifferentiated rat ES-like cells, we thus chose the rat PrE cells as a reference, in order to preclude those genes that are also expressed in the PrE cells. However, it is worth pointing out that some known pluripotency marker genes, such as *Klf4*, *c-Myc*, *Cdh1*, *Tbx3*, *Tbx15* and *Dppa3*, are expressed in both our rat ES-like cells and PrE cells. Thus, these genes are not included in our gene list of uniquely expressed genes in the undifferentiated ES-like cells when the rat PrE cells are used as a reference.

During our preparation of this manuscript, Ueda *et al.* [36] reported their establishment of rat ES cell lines, which could produce chimeric rats, as determined by PCR analysis of expression of a transfected GFP gene. By comparison, our study has the following advantages: i) our ES cell lines were each derived from the individual rat blastocyst, whereas the lines established by Ueda *et al.* were generated from the pooled cells of 42 rat embryos; ii) the antibodies used in our study were verified by embryo staining and our cells were alkaline phosphatase positive, which is a characteristic of both human and mouse ES cells, while the ES cells reported by Ueda *et al.* were alkaline phosphatase negative; iii) importantly, our cells reproducibly generated teratomas containing various tissues from all three germ layers. In contrast, their cells produced tumors containing tissues of only two germ layers, lacking ectodermal tissue. Production of teratomas containing tissues from all three embryonic germ layers is one of the essential criteria for characterization of ES cells from any species. In addition, we derived rat ES-like cells using a new medium which enhanced rat blastocyst attachment and the generation of Oct4-positive cells. Furthermore, we report a line of PrE cells derived from our established rat ES-like cells in this study. The PrE cell line provides a better reference

to identify genes and pathways specifically expressed in rat pluripotent cells, since PrE cells are the differentiated cell type most often seen in the culture of rat ES-like cells. In contrast, Ueda *et al.* used rat embryonic fibroblast cells as a reference; many endoderm marker genes were thus enriched in their rat ES cells. It should be pointed out that the PCR analysis used by Ueda *et al.* is not a rigorous verification of production of chimeric mice and that detection of GFP expression in various organs could have resulted from the presence of blood in the organs. Thus, more experiments are needed to confirm the generation of chimeric mice, especially given that their rat ES cells could not produce cell or tissue types from all three germ layers. Nevertheless, the two studies reveal that pluripotent embryonic cells can be generated from rat blastocysts under appropriate conditions and that the cell lines established independently share common properties, such as expression of pluripotency markers and Lifr, formation of EB in suspension culture and the ability to proliferate for an extended time *in vitro*. Both studies provide useful information for understanding the genetic basis of pluripotency and for optimization of rat ES cell culture conditions.

Materials and Methods

Animals and embryos

Rats of the Sprague-Dawley (SD) strain were purchased from Shanghai Laboratory Animal Center (SLAC) and maintained in SPF spaces with a 14-h light and 10-h dark regime. All animal procedures were performed according to the guidelines approved by the Shanghai JiaoTong University School of Medicine. Rat blastocysts of 4.5 dpc were flushed from the uterus with KO-DMEM (Gibco) supplemented with 40 mM HEPES (Gibco). Mouse and rat epiblasts were obtained from 6.0 dpc and 7.5 dpc embryos, respectively. MEF cells were prepared from ICR and KM strains at 13.5 dpc. For teratoma formation assays, 6- to 8-week-old SCID mice were employed.

Statistical analysis of attachment ratio

Attachment ratio of embryos was determined for whole and zona-pellucida free rat blastocysts. Whole rat blastocysts were plated onto irradiated mouse embryonic fibroblast (MEF) cells or matrigel with rat ES cell medium, which consisted of 50% mouse ES cell medium, 50% EScGRO (Millipore) and 2 μM of GSK-3β inhibitor (BIO, gift from Wei Han). To remove the zona-pellucida, whole rat blastocysts were treated with Tyrode's solution (Sigma) for 10 min at 37 ^{o}C. Zona-pellucida free rat blastocysts were then plated onto matrigel covered 4-well plates with different medium. Attachment ratio was calculated at different time points.

Derivation and culture of rat ES-like cells

Whole rat blastocysts were plated onto irradiated mouse embryonic fibroblast (MEF) cells. When ICM clumps appeared, rat ES-like cells were passaged mechanically in the early passages. From the sixth passage, 0.05% trypsin (Invitrogen) was used to passage the cells every four to five days. Mouse ES cell medium consisted of 90% DMEM (Invitrogen) supplemented with 10% FBS (Hyclone), 100U/ml penicillin (Invitrogen), 100 μg/ml streptomycin (Invitrogen), 0.1 mM β-mercaptoethanol (Gibco), 2 mM L-glutamine (Invitrogen) and 2 000 units/ml murine LIF (Chemicon). The rat ES-like cells were cryopreserved using 90% rat ES cell medium and 10% DMSO.

EB formation assays

EBs were generated by trypsinizing the rat ES-like cells and resulting single cells were cultured in suspension with EB culture medium, consisting of 90% DMEM, 10% FBS, 100 U/ml penicillin (Invitrogen), 100 μg/ml streptomycin (Invitrogen), 2 mM L-glutamine (Invitrogen) and 0.1 mM β-mercaptoethanol (Gibco). The medium was changed every other day.

Derivation and culture of rat PrE cells from rat ES-like cells

When rat ES-like cells were maintained on a feeder layer for more than seven days, endoderm-like cells appeared in the periphery of partially differentiated rat ES-like cell colonies, with the compacted undifferentiated rat ES-like cells in the center. We mechanically transferred the endoderm-like cells onto gelatin-coated culture dishes at a high density and cultured them in the mouse ES cell medium without LIF.

Teratoma formation assays

The rat ES-like cells were trypsinized and suspended at a density of 2×10^{6}/100 μl in rat ES cell medium. A total of 4×10^{6} cells were injected into muscle of the rear leg of 6-week-old male SCID mice.

Chimeric EB formation assays

The cells used in the assay were trypsinized and were dropped onto low attachment Petri dishes at a density of 300 cells/50 μl. Two days later, all the chimeric EBs were collected from the dishes and embedded in O.C.T. The PrE cells were labeled with EGFP for immunostaining of PrE markers. They were additionally labeled with a red fluorescent dye (DiI) to trace their location in chimeric EBs. The undifferentiated mouse ES cells (E14T, gift from Austin Smith and Ian Chambers) and the rat PrE cells were mixed at a ratio of 2:1.

Microarray analysis

For each sample, 5 μg of purified RNA was used for Affymetrix Chip analysis using GeneChip® Rat Expression Array 230 2.0. The computer data files to be used in data analysis (*.cel) were generated with the Affymetrix GeneChip Operating Software (GCOS) Version 1.4 (Affymetrix®), using the statistical algorithm provided. The data for the probe level intensity were then transferred to the ArrayAssist® Software (StrataGene; La Jolla, CA) for further analysis. For each sample, background was removed and data were normalized in accordance with the MAS 5.0. The genes whose expression was up-regulated more than 2-fold and Affymetrix "P" call in rat ES-like cells were selected as significant genes, when compared with rat PrE cells. Two biological replicates of the rat ES-like cells and the PrE cells were used to produce microarray data. The significant genes were analyzed in the context of the KEGG pathway in order to identify active pathways in rat ES-like

cells using DAVID 2008 (http://david.abcc.ncifcrf.gov/). For each term, the probability values were computed based upon a modified Fisher Exact test. EASE scores < 0.5 were considered as significant categories.

RT-PCR

Total RNA was extracted from cells using TRIzol (Invitrogen) and transcribed into cDNA using oligo(dT)15 and ReverTra Ace reverse transcriptase (Toyobo). PCR reactions were carried out as previously described [37]. The RT-PCR primers are shown in the Supplementary information, Table S3.

Immunofluorescence staining

The staining was performed as described previously [38]. All primary antibodies listed in the Supplementary information, Table S4 were diluted in the blocking buffer and incubated overnight at 4 °C. Fluorescently coupled secondary antibodies, including anti-rabbit FITC (Protein Tech Group, 1/100), anti-goat FITC (Jackson ImmunoResearch, 1/50), anti-rabbit Cy3 (Jackson ImmunoResearch, 1/300) and anti-mouse FITC (Jackson ImmunoResearch, 1/100), were incubated for 1 h at room temperature.

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(**Supplementary information** is linked to the online version of the paper on the Cell Research website.)