

Chapter 2

Establishment of ESC Lines Derived from Mice, Rats, and Primate

Abstract The first embryonic stem cell line was established from mice, which served as an important tool for ESC pluripotency and self-renewal studies. BN rat is the most widely used experiment model, it is important to generate the gene knockout animal model in BN rat for biology research. Gene modification in the ESCs is a common way to generate transgenic animal models. As a result, we established the ESC line from BN rat to facilitate the generation of gene knockout animal. There were large differences in ESC morphology and self-renewal regulatory signaling pathways between human and mouse ESCs. Hence, in this chapter, we also compared the processes to establish ESC lines from the embryo of human and mice respectively.

Keywords Mice ESC • Rat ESC • Human ESC • Self-renewal • Pluripotency

2.1 Introduction

In 1981, Evans et al. established the first mouse embryonic stem cell line from the strain 129SvE. In the same time, Martin et al. generated the embryonic stem cell line from strain ICR *SWR/J used the condition medium from teratoma stem cell. Embryonic stem cell from mice is an important model to explore the mechanisms of pluripotency and self-renewal. Additionally, it serves as a reference for embryonic stem cell study for human and other species.

LIF and BMP signaling pathways play the principal roles in maintaining the self-renewal state of mouse ESCs. These extracellular signals activate the expression of the master pluripotent genes such as Oct4, Sox2, Nanog in mouse ESCs and maintain the mouse ESCs at an undifferentiated state. Mouse ESCs also express genes such as FGF4, which can activate the downstream target pathway ERK1/2 to make the ESC differentiate to neuron lineage. BMP4, or similar proteins, existing in the mouse culture medium can inhibit the differentiation induced by FGF. Additionally, adding the inhibitor of ERK1/2 pathway can also inhibit ESC differentiation, and its function is independent of the LIF pathway.

Mouse ESCs can differentiate into three germ layers in embryoid bodies (EB), when LIF is withdrawn. The undifferentiated ESCs can integrate into all germ layers of the chimeric animals, including the genital ridge. When mouse ESCs were injected into the tetraploid blastocysts, mice could be generated from all injected ESCs, and this is the highest standard to identify the pluripotency of pluripotent stem cell; until now, no successful case has been reported in other animals for tetraploid complementation experiment.

Although mice are used as the most important human disease model, rat is better for some complicated disease studies such as nervous system and cardiovascular diseases. In 2008, rat ESCs were established successfully. Ying et al. were the first to report that P53 knockout rats were generated successfully based on rat ESCs. This confirms that we can generate genetic defective rats by knockout technique. BN rat is the most widely used rat, and has already been sequenced in whole genome wide, so it is an ideal animal model for human diseases. However, ESCs from BN rats have not been established yet, and it is still to be known whether BN rat ESCs can be established through the current method. Additionally, it also needs to be known whether one can generate rat ESCs with higher pluripotency, and whether these ESCs can be used to generate rat through tetraploid complementation.

The self-renewal of rat embryonic stem cells needs more stringent conditions. It needs to inhibit ERK signaling, and activate WNT signaling and LIF pathway to maintain a metastable pluripotent state. Unlike embryonic stem cells from mice and rats, human embryonic stem cells need to activate ERK1/2 signaling, but not LIF signaling, to maintain self-renewal. It is not clear whether these differences result from their different species origins; another explanation could be that the present human embryonic stem cells are not real naive embryonic stem cells.

In 2007, scientists isolated the epiblasts from early implanted embryos, and then established a stable stem cell line called EpiSCs. They need to activate ERK1/2 signaling to maintain their self-renewal state, and do not rely on LIF signaling pathway, similar to human ESC. In addition, the morphology as well as the gene expression of EpiSC is similar to human ESCs, but quite different from mice ESCs. EpiSCs have poor ability to form a chimera mouse, and are unable to integrate to the genital ridge. In 2009, scientists cultured mouse blastocysts in the human ESC environment, and then separated the cells called Fab-SCs. These cells show similarity to EpiSCs in signaling pathways regulation, morphology, and differentiation tendency, but are different from mouse ESCs. Because of the similarity among human ESCs, EpiSCs, and Fab-SCs, a debate over the nature of human ESCs was initiated recently.

As early as 1998, human embryonic stem cells were successfully derived. It was an important model for the current human embryonic developmental researches, and it is also an important cell source in regenerative medicine (Thomson et al. 1998). Human embryonic stem cells can differentiate into a variety of tissues and cells, which could be used for the treatment of different diseases such as Parkinson's disease, spinal cord injury, and some untreatable diseases nowadays. It is also an important disease model widely used in drug development. However unlike mouse ESCs, it is difficult to generate human transgenic ESCs because of low survival ability for single cell passage and due to difficulties in operating and amplification.

Experience from studies on mice cannot be applied to studies of human ESCs. These barriers will hinder the application of human ESCs in clinical applications. Therefore, obtaining human embryonic stem cells with totipotency will greatly promote the research and application of human ESCs.

In this chapter, we establish embryonic stem cells with pluripotency from the embryos of mouse BN rats, and human. The establishment of embryonic stem cells will build a stable foundation for the future study of totipotency in human ESCs.

2.2 Materials and Methods

2.2.1 The Experimental Animals

The CD-1, 129S2 mice, and BN rats were purchased from Beijing Vital River Labs. C57/B6, DBA2 mice, and B6D2F1 (C57BL/6j × DBA/2j) were obtained by breeding by laboratory technicians. All animal operations follow the laboratory animal management regulations of Beijing.

2.2.2 Discarded Embryos

Discarded embryos were provided by the reproductive medicine center of Peking University Third Hospital. They have no value for cryopreserve. All embryo providers signed the informed consent, and operation followed the laws and regulations of the Beijing human embryonic experiment ethical management committee and the Beijing government.

2.2.3 Generation of Mouse Embryonic Stem Cells

Mouse embryonic stem cells were established through the methods reported previously (Zhao et al. 2007). Briefly, E3.5 mouse embryo were collected and digested with 0.5 % Pronase E (Sigma) for 5–10 min. The zona pellucida were removed and cultured for 6–9 days before picking the outgrowth. The outgrowths were replated in a petri dish with feeder cells.

2.2.4 Generation of Rat Embryonic Stem Cells

Generation of rat embryonic stem cells was referred to Li and other's reports (Li et al. 2008). The brief introduction is as follows: Briefly, E4.5 rat embryo is collected and digested with 0.5 % Pronase E (Sigma) for 3–10 min. The zona pellucida were removed and cultured in a petri dish with the feeder layer for 4–6 days before picking the outgrowth.

2.2.5 Generation of Human Embryonic Stem Cells

Method of generation of human embryonic stem cells is briefly introduced as follows: The zona pellucida of the E6 or E7 embryos were removed and, plated in a petri dish with feeder cells for 4–10 days before picking the outgrowth. Routinely picked the clones before 10 passages.

2.2.6 Karyotype Analysis

0.05 % democosin drugs (Sigma) were added in the culture medium to treat the embryonic stem cells for 2–3 hour. The ESC clones were digested into single cell and collected, and were incubated at 37 °C in 0.56 % KCL solution for low permeability for 20 min. Cells were centrifuged to collect and treated with fresh fixed liquid mixture (methanol: acetic acid = 3:1) for 30 min. Cells were centrifuged to collect and treated with fresh fixed fluid mixture (methanol: glacial acetic acid = 1:1) at room temperature for 30 min. Cells were centrifuged to collect and resuspend with 200 µl fixed liquid. 15–20 µl cell solution was taken for one drop at a time. After the slides were dried, Giemsa (Sigma) was used for dyeing for 7–10 min. After rinsing with water, slides are observed under a microscope. The metaphases sample with no cross-overlapping between chromosomes was selected for counting. Each metaphase sample was counted thrice. 50–100 metaphases were taken randomly for counting.

2.2.7 Alkaline Phosphatase Staining

Alkaline Phosphatase staining followed the instructions of Beyotime BCIP/NBT Alkaline Phosphatase Color Development Kit (Beyotime) specifications. Cells fixed with 4 % paraformaldehyde are stained with BCIP/NBT for 15–30 min, and then observed under the microscope.

2.2.8 Immunofluorescence Staining of Embryonic Stem Cells

Removed the culture medium from the dishes and washed the cells with PBS 3–5 times. Used 4 % polyoxymethylene to fix cells at room temperature for 30 min, then added 0.5 % Triton–PBS to permeabilize for 30 min. After several washes, cells were blocked in 2 % BSA–PBS for 1 h at room temperature and then incubated at 4°C overnight with primary antibody. The primary antibodies were Oct4 (10, Santa

Cruz), Sox2 (Chemicon), Nanog (Chemicon), SSEA-1 (Chemicon), SSEA-3, SSEA-4, Tr-1-60, and Tr-1-81. The cells were then washed with PBS, and secondary antibodies were added. Cells were incubated at room temperature for an hour. After wash, 10 $\mu\text{g/ml}$ PI (10 $\mu\text{g/ml}$; Molecular The Probes, OR, USA) was added at 37 °C for 20 min or 10 $\mu\text{g/ml}$ Hoechst was added for 15 min. The slides could be conserved in -20° . All the samples were observed with Zeiss LSM 510 META laser confocal (German) Scanning microscope.

2.2.9 RT-PCR Detection of Gene Expression in Embryonic Stem Cells

Embryonic stem cells were collected after feeder cells were withdrawn. Washing cells with PBS twice, added Trizol (Invitrogen) to extract RNA, and used isopropyl alcohol to precipitate total RNA. Used SuperScript III Reverse Transcriptase (Invitrogen) and oligo-dT (Promega) to reverse transcript cDNA as instructed. qPCR experiments using SYBR green (Sigma) reagents were performed on ABI 7000 quantitative PCR.

2.2.10 Bisulfite Treatment

Bisulfite treatment was according to the EpiTect Bisulfite Kit (Qiagen) Kit instructions. 2 μg genome DNA from embryonic stem cells was taken for bisulfite treatment. After treatment, samples were stored in -20° freezer and used for nest PCR reaction to amplify the methylation fragments of Oct4 and Nanog promoter region. The first and second rounds of PCR reaction conditions were the same: 94 °C for 5 min. 35 cycles (59 °C for 30 s, 94 °C for 45 s, and 72 °C for 30 s). The last 72 °C extended for 7 min. The PCR products were cloned to PMD18-T carrier. 15 samples were picked randomly for cloning sequencing.

2.2.11 Teratoma Formation

3×10^6 vigorous growing embryonic stem cells without feeder cells were injected subcutaneously into the severe combined immunodeficiency (SCID) mice. After 2–5 weeks, formation of teratoma was observed. Mice were killed by breaking the neck, and teratoma was taken out, weighed, and pictured. A small amount of abnormal fetal tumor tissues were fixed in 4 % paraformaldehyde, embedded in paraffin, and sectioned. After HE, staining was applied on sections and slides were observed under the microscope.

2.2.12 Blastocyst Injection (Chimeras and Tetraploid Complementation)

Putting the blastocysts (E3.5 for mouse blastocysts, E4.5 for rat blastocysts) under the platform of the inverted microscope. 8–12 embryonic stem cells were injected into the blastocoels. One hour after operation, the embryos were transplanted into pseudopregnant uterus of mother rats. 18 or 21 days later, the pseudopregnant mothers were killed under Caesarean section. Tetraploid complementation was similar to the diploid blastocyst injection. E1.5 mouse embryos at the two-cell stage were electrofusion to gain tetraploid embryos, and continued to culture to the expanded blastocysts stage. 10–15 mice embryonic stem cells were injected into the blastocoels. After a short recovery, the embryos were transplanted into the pseudopregnant mice. The pseudopregnant mice were killed under Caesarean section for the following observations.

2.3 Results

2.3.1 Production of Live Mouse Derived from Mouse ESC with Tetraploid Embryo

We obtained 27 mice blastocysts by intracytoplasmic sperm injection (ICSI) (B6D2F1, C57BL/6j $\times$ DBA/2j), and cultured them for about 5–7 days. We obtained 14 outgrowths (Efficiency, 14/27, 52 %), randomly selected 11 outgrowths, and established 11 embryonic stem cell lines, including four cell lines carrying Oct4-GFP report gene, which could indicate whether Oct4 is activated under fluorescence. In the majority of the differentiated embryoid bodies, no GFP can be detected. We randomly selected one cell line (ESC2) for further experiments. Bisulfite sequencing results showed that Oct4 and Nanog promoter regions are hypomethylated. Immunostaining proved that ESC2 expressed pluripotent stem cells surface markers SSEA-1, as well as core pluripotency factors Oct4 and Nanog. After injecting ESC2 into SCID mice, teratoma was observed after 3 weeks. After HE staining, we found that embryonic stem cells differentiated to form all three layers of tissues, which suggested that ESC2 was the pluripotent stem cell.

We selected ESC2 for the next experiment. First, we did the tetraploid complementation experiment. 1,092 embryos were injected, and 34 mice were born through tetraploid complementation. A few mice could survive to adulthood and reproduce the next generation. Then, we tested the diploid chimeric ability of the ESCs. The results showed that the cell line has a strong ability to form chimera. We also tested ESCs from other genetic backgrounds. Two of three mouse ES cell lines with 129S2 background can generate full term mice (Fig. 2.1), and two of three mouse ES cell lines with F1 genetic background (C56BL/6J $\times$ 129S2) can generate mice.

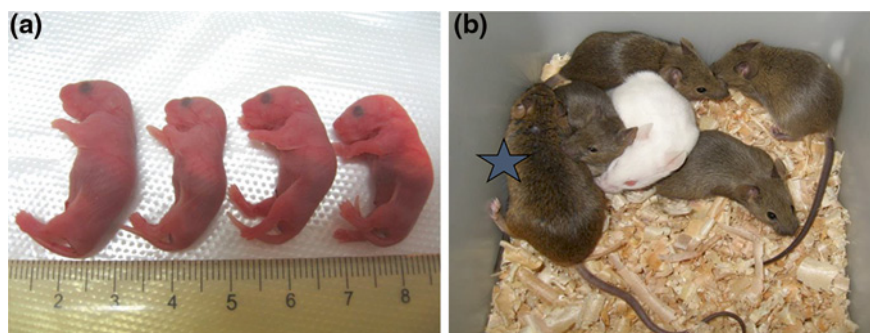


Fig. 2.1 Generation of ES mice by tetraploid complementation. **a** Four newborn ES mice. **b** Pups were produced by the ES mice mated with wildtype mice

The above data shows that our system to establish ESC lineage was reliable and a high ratio of ESC has the ability to generate mice through tetraploid complementation experiment. These mouse ES cell lines are pluripotent stem cells verified by the highest standard. Thus, these cell lines can be used to study the mechanisms of self-renewal and pluripotency, and as control for the following iPSC experiments.

2.3.2 Establishment of Rat BN ESC

After generating mice ES cell lines with the ability to generate mice through tetraploid complementation, we started to establish rat ES cell lines. We obtained E4.5 blastocysts from the uterus of BN rats. After removing the zona pellucida, the embryos were cultured on the feeder cells in four well dishes in an N2B27 culture medium with 2 I (1 $\mu\text{mol/L}$ PD25901, 3 $\mu\text{mol/L}$ CHIR99021) and LIF for 4–6 days. Then, a total of four outgrowths were obtained. Using 10 μl pipette, we picked the outgrowths out, and digested cells with 0.05 % trypsin to small clumps, then used serum to stop the digestion and passaged to the new well to continue the culture. Two stable BN rat ES cell lines were finally generated. These cell lines had been passaged for 30 generations, without obvious differentiation (Fig. 2.2). All of the embryonic stem cell lines used in the following experiments were between 5 and 30 passages. At the same time, we established four ES cell lines from SD rats, and four ES cell lines from rats mated between BN and F344 background. The range of efficiency for all the ES cell lines was from 2 to 47 %.

We first analyzed the karyotype in the metaphase of our BN rat embryonic stem cells. More than 70 % of the cells have 42 chromosomes, suggesting that the two BN–ES cell lines are of normal karyotype. Through alkaline phosphate enzyme staining (AP Staining) we found that BN–ESCs are AP positive. Further RT-PCR analysis suggested the BN–ES cell lines expressed many pluripotency

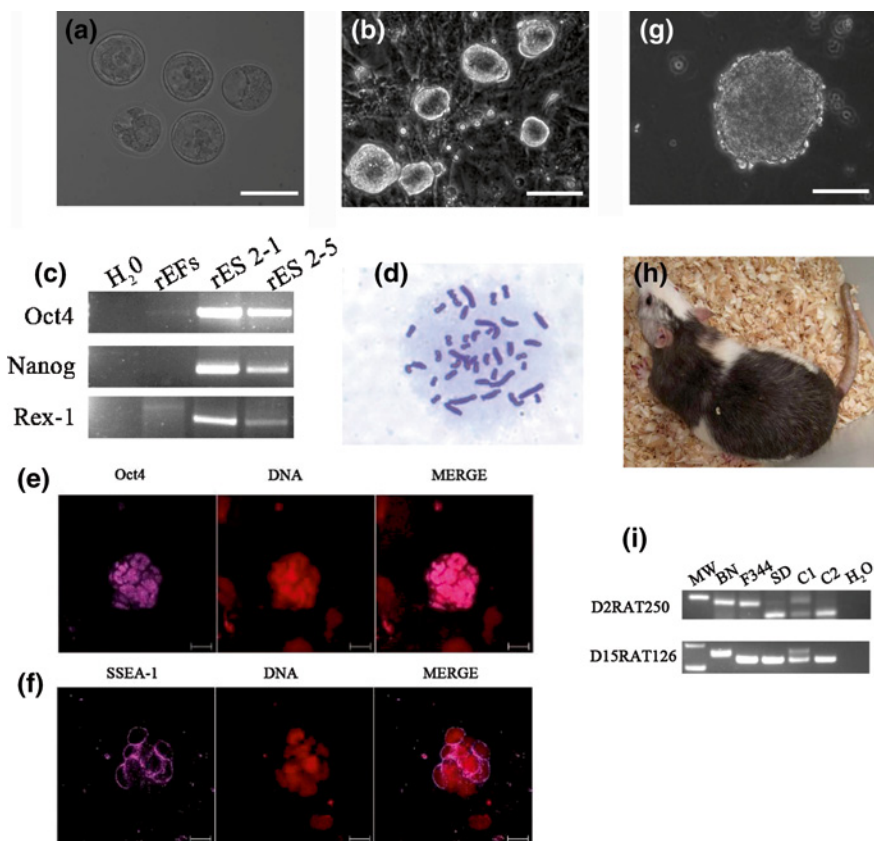


Fig. 2.2 Derivation of pluripotent stem cell lines from blastocysts of Brown Norway (BN) rats. **a** Blastocysts of BN rat. **b** Cell morphology. BN rat ES cells rES 2-1 (passage eight). **c** RT-PCR analysis of the rat pluripotent stem cells. H₂O is negative control, rEFs are rat embryonic fibroblasts, rES 2-1 and rES 2-5 are two rat ES cell lines. **d** Karyotype of rES 2-1 rat ES cells (passage eighteen). Bar = 100 μ m. **e** Immunostaining of the pluripotency markers Oct4 (pink) in rat ES cells rES 2-1 (passage ten). **f** Immunostaining of the surface marker SSEA-1 (pink) in rat ES cells rES 2-1 (passage ten). Bar = 50 μ m. **g** Embryonic bodies (EBs) formation of the rat ES cells. Bar = 100 μ m. **h**, Chimeric rats generated by injecting BN rat ES cells rES 2-1 (brown color) into SD blastocysts (white color). **i** SSLPs analysis of the chimeric rats. MW is molecular weight, H₂O is negative control, BN, F344, SD are tail tips from the BN, F344, SD rats, respectively, C1 is tail tips from the chimeric rats with 75 % chimerism, C2 is tail tips from the brothers of C1 with ≤ 0 % chimerism. Reprint from Zhao et al. (2007) Co-published by Elsevier Ltd. and Science Press in China. © Genetics Society of China and Institute of Genetics and Developmental Biology. Reprinted with permission.

factors such as Oct4, Nanog, Rex-1, and so on. Immunostaining results showed that BN-ES cell lines expressed Oct4 and SSEA-1. When we removed 2i from the BN-ESC culture medium, it could form the embryoid bodies after 4 days.

Although the rat ESCs grew rapidly, the clones showed good three-dimensional morphology. However, ESCs from rats and mice still have some subtle difference. Compared to rat ESCs, mouse ESCs were easier to form embryoid bodies when LIF was withdrawn from the medium. If rat ESCs were cultured in the medium

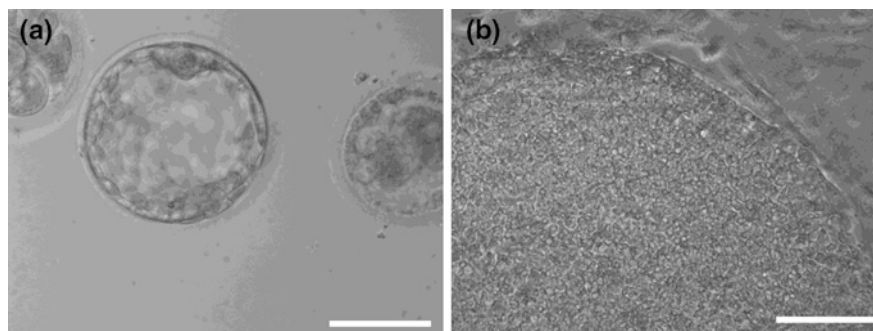


Fig. 2.3 Derivation of embryonic stem cells from poor-quality human blastocysts. **a** E7 blastocysts. **b** Stabled human ESC of passage 6

containing serum, they usually died, instead of differentiation, so it was difficult to form embryoid bodies from rat ESCs. The reason for this phenomenon is not clear.

We chose one BN-ES cell line for the *in vivo* experiment to detect pluripotency. When injecting the BN-ESCs into E4.5 SD blastocysts, we obtained four surviving chimeric rats (2 males and 2 females). The chimerism is between 60 and 80 %. Then we analyzed the single nucleotide polymorphisms of chimeric rats, and the result proved that these chimeric rats came from BN-ESC cells. When the chimera rats were mated with wildtype SD rats, we did not get rats with germline transmission (Zhao et al. 2010).

2.3.3 Generation of Human ESC

After generating ES cell lines from mice and rats, we began to study human embryonic stem cells. We collected the E3.0 discarded embryos and cultured them for 3–4 days, and then obtained a small amount of blastocysts. The majority of the embryos were of low quality. They did not have the inner cell mass, or the inner cell masses were not clear. After enzyme digestion and zona pellucid removal, we plated the blastocysts on the feeder cells in the four well dishes for 4–10 days. Then, we used a glass needle to pick the outgrowths out, and plated cells into a new dish for 7–10 days. Then, we used the glass needles to cut the outgrowths into several pieces and plated in the new dishes. After 2–3 repeats, we obtained stable cell clones.

From 37 blastocysts, we acquired four outgrowths (4/37, 10.8 %), and finally successfully established three ES cell lines, named BHE-1, 2, and 3. The other outgrowth differentiated after the first picking. Human embryonic stem cells grew in monolayer, which could be easily distinguished from feeder cells. The growth speed of human ESCs was slower than that of mouse ESCs, and cells were passaged every 5–7 days (Fig. 2.3). Human ESCs depended on the FGF2 pathway to maintain their undifferentiated state. When FGF2 was withdrawn, the human ESCs differentiated rapidly. Among these three human ES cell lines, BHE-3 grew

the best while the other two cell lines differentiated slightly during growth. This suggested that different human ES cell lines showed different properties, which was consistent with the previous reports (Chung et al. 2008).

2.4 Discussion

2.4.1 *Significant Difference Between Mouse and Rat ESCs*

LIF/Stat3 pathway, BMP pathway, and WNT pathway are the major signaling pathways involved in mouse ESC maintenance. Previously, scientists just needed to add LIF and serum in the medium to maintain mouse ESCs in an undifferentiated state. BMP signaling pathway was considered to play a role in mouse ESC self-renewal. We still have some controversy about the origin of mouse ESCs. Some scientists believe that mouse ESCs are induced by the culture environment around the cells, especially the LIF, BMP, and other factors added into the medium, while others believe that the pluripotency state of mouse ESCs is equal to a developmental stage in vivo. In 2007, Kunath found that when they inhibit the FGF signaling pathway, which can induce mouse ESCs to differentiate, mouse ESCs can maintain self-renewal (Kunath et al. 2007). In 2008, Yingqi Long et al. reported that if they inhibited ERK1/2 pathway and activated WNT signaling pathway at the same time, the inner cell mass isolated from the mice blastocysts can maintain their undifferentiated state, and establish a stable ES cell line (Ying et al. 2008). The conclusion of these studies is that mouse ESCs is equal to E3.5–E4.5 epiblasts with similar characteristics.

In 2008, based on their previous work on mouse ESC generation, Buehr established the rat ES cell line, which had the ability to generate chimeric rats. Their work fulfilled the desire of researchers to generate rat ES cell line since the past decades. The signaling pathways that are involved in self-renewal of rat ESCs included ERK1/2 signaling, which should be inhibited, and WNT and LIF/STAT pathways, which should be activated. However, rat ESCs are easier to differentiate during culture, and the clones cannot grow big. These observations suggest that the culture medium for rat ESCs need to be modified, and scientists were not very clear with the mechanisms to maintain the self-renewal of rat ESCs. Additionally, it is not clear whether rat ESCs can differentiate into all cell types, and we cannot generate the rats through tetraploid complementation.

BN rat ESCs can be established, and the efficiency is not varied between BN rat and other strains. This suggests that the genetic background of rats has limited impact on ESC generation efficiency, which is different from mice. When BN rat ESCs were injected into SD rat blastocysts, highly chimeric offsprings could be generated. However, they do not have the ability to generate the chimera with germline transmission. This result is consistent with Li's report, which showed that blastocysts from SD rat are not suitable to be used as receptor blastocysts. The next step of our work is to use blastocysts from F344 as the receptor blastocysts to test the pluripotency of BN rat ESCs.

2.4.2 Difference Between Mouse ESC and Human ESC

Although mouse and rat ESCs have some similarities in the mechanisms to maintain self-renewal, there are still some differences. Obviously, gene expression pattern and the mechanisms to maintain self-renewal in human ESCs and rodents ESCs showed significant differences. Human ESCs need FGF2 to maintain undifferentiated state, and activin/Nodal and WNT signaling pathways to maintain self-renewal (Potzi et al. 1991; Rajala et al. 2007; Richards et al. 2002; Sato et al. 2004; Xu et al. 2005). They do not need LIF and BMP signaling (Xu et al. 2002). Compared to mouse ESCs, the survival rate and growth speed for single human ES cell is low. For these reasons, genetically modified human ESC is difficult to generate, which has prevented human ESCs to be used as research tool. In addition, the evaluation standards for human ESCs are conventional, lacking in functional verification. The differences among various human ES cell lines are also large. Therefore, if we can get a human ES cell line with pluripotent state similar to mouse ESCs (naïve-ESC) (Ren et al. 2009), the research results in mouse ESCs will be applied to human ESC studies, which will greatly accelerate the application of human ESC. In addition, in-depth researches can be conducted conveniently with easier manipulation of human ESCs.

Nowadays, scientists are still confused in understanding the origin of the differences between human ESCs and mouse ESCs. Species difference is the important reason. Even mouse and rat, both of which belong to rodent animal, use different mechanisms to regulate self-renewal. Thus, we can speculate that human ESCs and mouse ESCs must have more differences. Because the human inner cell mass is different from that of mouse, ESCs isolated from blastocysts must show some different characteristics and thus, the differences between ESCs originated from species differences.

In 2007, scientists isolated epiblasts from implanted E5.5–E7.5 mouse embryos, and obtained the epiblast stem cell (EpiSC) after culture. EpiSC and human ESC have some similarities in morphology, gene expression, signaling pathways involved in self-renewal, etc. By contrast, it is greatly different from mouse ESCs. Thus scientists began to speculate that the big difference between human and mouse ESCs is caused by the different developmental stage, and hence human ESCs with naïve state can be isolated in the future.

However, both the theories mentioned above lack key evidence. As it is difficult to obtain human embryos, scientists can use only a limited number of embryos that are not good in the morphology for research. Thus, it is hard to make a breakthrough in this field, and will restrict the generation of human naïve ESCs. So it will be more important to study the mechanisms to maintain self-renewal in mice and rats. It is also valuable to use ESCs from other species as a model for research, such as nonhuman primates. In addition, iPSC can be another alternative choice.

The combination of inhibitors for GSK3, ERK1/2, and TGF-beta signaling can induce fibroblasts to form human naïve iPSCs, which have similarity with mouse

ESCs in morphology (Li et al. 2009). Continuous expression of Oct4, Sox2, Klf4, coupled with the administration of Fostolin and inhibitors for GSK3 and ERK1/2 inhibitors can also be used to obtain the naive human iPSCs, which had the activated X chromosome and showed more similarity with mouse ESCs (Hanna et al. 2010). Through simply expressing Oct4, Sox2, Klf4, c-Myc, and Nanog, human fibroblasts can be induced to naive iPSC. This kind of iPSC is not stable, and requires the continuous expression of exogenous factors. After the removal of exogenous factors, naive iPSC would differentiate into the prime-ESC, which is similar to human ESCs (Buecker et al. 2010). In addition, by adding small compounds, which can change human ESC epigenetic state, or ROCK inhibitors, a pluripotent stem cell can also be obtained that have characteristics between naive-ESC and Prime-ESC state (Ellerstrom et al. 2006; Xu et al. 2010). These results show that there may be naive human ESCs, which may be in the sub-stable state and need new signaling pathways to maintain their state of self-renewal. So, we may need to research for a long time in order to solve these scientific questions.

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