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LETTER TO THE EDITOR

Derivation of Germline Competent Rat Embryonic Stem Cells from DA Rats

The laboratory rat was one of the earliest mammalian species for scientific research and used as animal disease models in physiology, toxicology, behavior, immunology, and tumor-biology for over 150 years (Jacob, 1999). However, rat lags far behind mouse in generating human disease models and functional genomic studies because of the lack of authentic rat embryonic stem (ES) cells (Voigt and Serikawa, 2009), whereas the first mouse ES cell line was established in 1981 (Evans and Kaufman, 1981). By combining two or three kinase inhibitors which target GSK3, MEK and FGF signaling pathways in serum-free N2B27 medium, germline competent rat ES cells were first derived in 2008 (Buehr et al., 2008; Li et al., 2008). Soon after, the first knockout rat *via* ES cell-based gene targeting technology was reported, which was generated by disruption of the tumor suppressor gene *Tp53* in rat ES cells and generated *Tp53* gene knockout rats by means of homologous recombination (Tong et al., 2010). Subsequently, another report showed the production of a protease-activated receptor-2 gene (*Par-2*) knockout rat with a similar method (Yamamoto et al., 2012). Furthermore, Zinc-Finger Nucleases (ZFNs) mediated homologous recombination was reported in the generation of knockout and knockin rats (Geurts et al., 2009). However, ZFNs technique has not yet succeeded in conditional genetic manipulations, such as tissue- and time-specific knockout/knockin.

In China, stem cell research is becoming one of the most popular scientific fields (Dai and Gao, 2010), and the research of rat ES cells advanced quickly. Followed the first derivation of rat ES cells from DA rat strain, our group has successfully derived ES cells from Brown Norway (BN) rat strain (Zhao et al., 2010). Rat ES cells resemble the mouse ES cells as they could differentiate into functional cardiac cells *in vitro* (Cao et al., 2011). Recently, rat ES cell clones with different morphology termed d-rESCs and f-rESCs under the 2i and Lif condition were reported, which indicated that the heterogeneous characteristics of rat ES cells might be correlated with the differential gene expression profiles (Shen et al., 2011). Rat induced pluripotent stem cells (iPSC) were reprogrammed by forced expression of the Yamanaka factors (Oct4, Sox2, Klf4, and c-Myc) in adult rat somatic cells (Liao et al., 2009). However, so far there was no report on the derivation of germline competent rat ES cells in China. This may be caused by the culture system instabilities, which may restrict the application of ES cell-based rat disease models in the future.

It was reported that under the culture condition of DMEM medium containing 20% fetal bovine serum (FBS) supplemented with 2i (inhibitors PD0325901 and CHIR99021) and two other inhibitors Y-27632 and A-83-01 (YPAC), rat ES cells were established, by which gene modified rats were generated (Kawamata and Ochiya, 2010). Therefore, we hypothesized that rat ES cells could be more stable when cultured with serum-free N2B27 medium supplemented with 2i combining inhibitors Y-27632 and A-83-01 (termed N2B27-4i), which may improve the efficiency of chimera rat production. To test this hypothesis, we derived DA rat ES cells first and then checked whether N2B27-4i medium could support generating germline transmission chimeras or not.

We derived rat ES cells from the inner cell mess (ICM) of E4.5 dpc (days post coitum) DA blastocysts. A total of 12 blastocysts were flushed from uterus of pregnant DA rats (Fig. 1A), whose zona pellucidae were removed and then placed on 4-well plates coated with feeder cells, containing N2B27-2i medium. After 4−6 days culture, blastocysts attached and the outgrowths, a prominent central mass of cells, appeared. After picked out and disaggregated into single cells suspension with 0.25% trypsin-EDTA, resuspended in rat ES cell medium and plated in a new 4-well plate, the rat ES cell colonies were maintained for later research. As a result, a total of 10 ES cell lines were derived from the 12 blastocysts plated, with an efficiency of 85% (Table S1). The morphology of the rat ES cells derived was compacted colony, similar to the mouse ES cells (Fig. 1B and C). All rat ES cell colonies were positive for AP staining (Fig. 1D). Karyotype analysis of the rat ES cells was also carried out with a result of a predominantly normal diploid with 42 chromosomes (Fig. 1E). All rat ES cell lines cultured in N2B27-2i medium maintained an undifferentiated state for over 30 passages.

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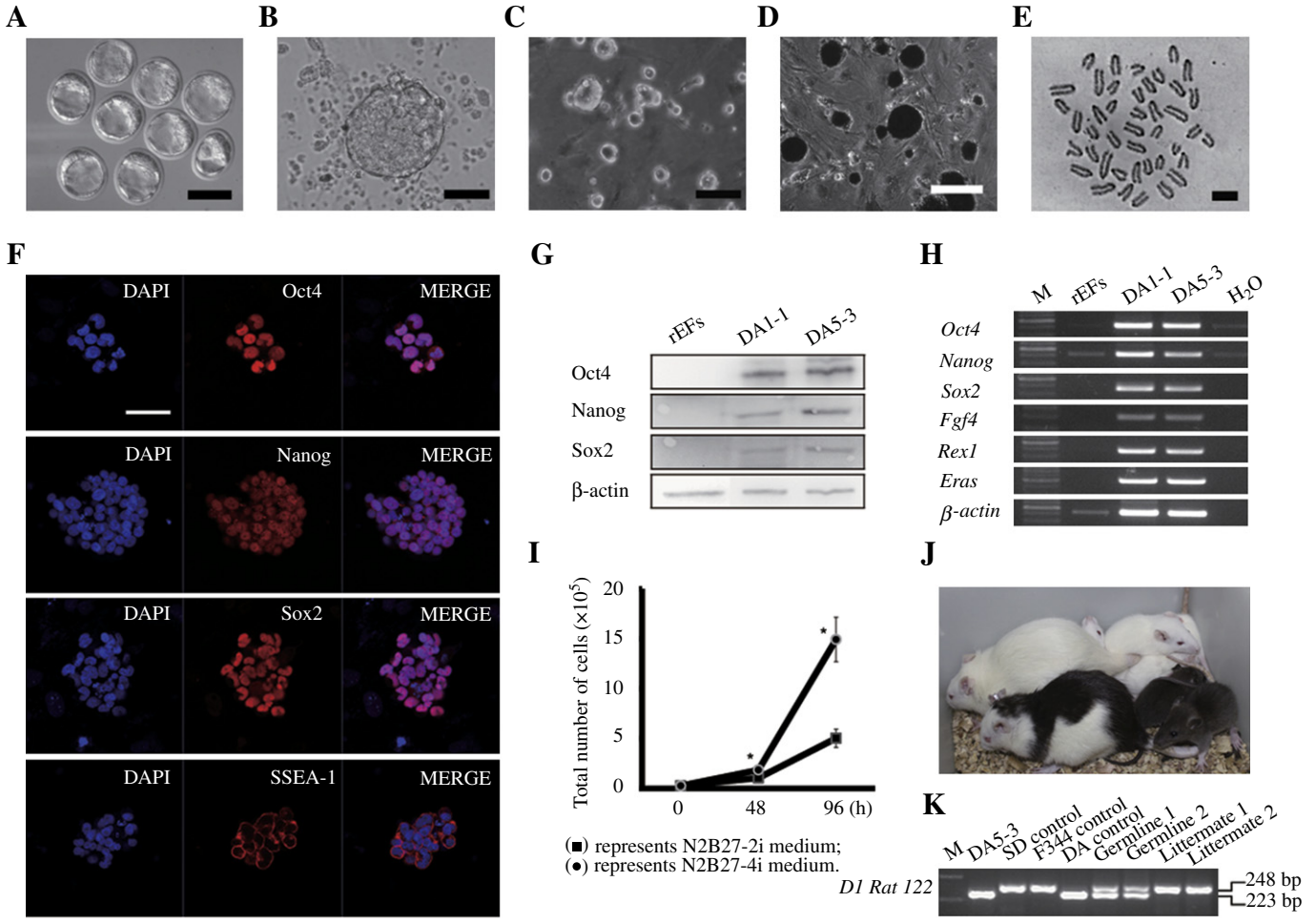

Fig. 1. Derivation and characterization of germline competent DA rat ES cells.

A: E4.5 blastocysts flushed from uterus of pregnant DA rats. The scale bar = 100 μm. **B:** outgrowths attached to feeders after 4 days of blastocysts plated. The scale bar = 100 μm. **C:** cell morphology of DA5-3 rat ES cells (passage 8). The scale bar = 100 μm. **D:** alkaline phosphatase (AP) staining of DA5-3 (passage 8). The scale bar = 100 μm. **E:** karyotype of DA5-3 rat ES cells (passage 8), indicating a chromosomal number of 42, XX. The scale bar = 10 μm. **F:** immunofluorescence staining for Oct4, Nanog, Sox2 and SSEA-1 in DA5-3 rat ES cells (passage 8). The scale bar = 100 μm. **G:** Western blot analysis of Oct4, Nanog, Sox2 expression in two rat ES cell lines and rat embryonic fibroblasts (rEFs) (control). **H:** RT-PCR analysis of pluripotentcy marker of *Oct4*, *Nanog*, *Sox2*, *Fgf4*, *Rex1*, and *Eras* expression in two DA rat ES cell lines, rEFs and H_2O (control). M, 100 bp DNA marker. **I:** total number of rat ES cells was counted at the point of 0, 48 and 96 h after 2.5×10^4 DA5-3 cells passaged, cultured with N2B27-2i or N2B27-4i medium. The superscript (*) represents significant difference ($P < 0.05$). **J:** the germline offspring produced by the female chimeric rat mated with an SD male, two germline offspring (brown coat) in nine pups were shown. **K:** simple sequence-length polymorphism (SSLP) analysis of DA rats ES cells, germline offspring and littermates. M, 100 bp DNA marker.

To characterize the rat ES cells, we detected the expression of pluripotency markers. RT-PCR data showed that the rat ES cells expressed *Oct4*, *Nanog*, *Sox2*, *Fgf4*, *Rex1* and *Eras* (Fig. 1H). Immunostaining and Western blot data showed that the rat ES cells expressed Oct4, Nanog, Sox2 and SSEA-1 (Fig. 1F and G), which further confirmed the pluripotent state of the ES cells. In addition, the rat ES cells differentiated to EBs (Fig. S1A) and further differentiated into neural stem cells (NSCs) after plating in neural induction medium *in vitro* (our unpublished data).

To evaluate the pluripotency of rat ES cells further, 10−15 DA rat (brown coat) ES cells were injected into each diploid F344 (white coat) blastocyst to produce chimeric rat. As a result, chimeric rats were obtained from two rat ES cell lines (DA1-1, DA5-3). Simple sequence-length polymorphism (SSLP) analysis showed the contribution of rat ES cells in these chimeric rats (Fig. S1C). Some chimeric rats showed over 60% chimerism in the coat colour and a portion of chimeric rats could survive to adulthood (Fig. S1B). To determine whether these rat ES cells could contribute to germline, chimeric rats were mated with SD rats of white coat. We obtained offspring with brown coat colour from three parental chimeric rats generated from the rat ES cell lines injected at passage 7−10 (Fig. 1J). SSLP analysis confirmed that these offspring with brown coat inherited from both DA rat and SD rat (Fig. 1K). All these data indicated the rat ES cells we derived could produce chimeric rats and also contribute to the germline.

To test whether N2B27-4i medium was an optimal system for culturing rat ES cells, we cultured rat ES cells and maintained for more than 30 passages. It was interesting that N2B27-4i culture medium could enhance rat ES cells proliferation, and the total number of the cells cultured in N2B27-4i

Table 1
Generation of chimeras and germline competent offspring from rat ES cells

Medium	Cell line	Passage number	No. of blastocyst injected	Development of injected F344 blastocysts (number)			
				Pups	Chimeras	Adults	Germline
N2B27-2i	DA1-1	7, 8,10	102	35	23	4F/8M	2F
	DA5-3	6, 7	123	12	5	1F/2M	1F
	DA5-3	20, 21	186	11	3	1F/1M	None
N2B27-4i	DA5-3	7	167	23	17	3F/8M	2F
	DA5-3	30, 32	54	18	14	5F/9M	2F

F, female; M, male. Rat ES cell lines DA1-1 and DA5-3 were both derived in N2B27-2i medium; two kinds of medium, N2B27-2i and N2B27-4i, were used for rat ES cell long-time culture.

was approximately two times as high as that in N2B27-2i at the time point of 96 h (Fig. 1I). Furthermore, we injected these extensively passaged rat ES cells cultured with N2B27-4i to F344 blastocysts to determine their ability of generating chimeras and their capability of germline transmission. As expected, we obtained 17 chimeras from 167 injected blastocysts and 2 germline transmission rats by using the ES cell line DA5-3 at passage 7. Surprisingly, we also obtained germline transmitted chimeras from rat ES cell lines cultured in N2B27-4i medium even with high passages such as 28−32, whereas it was difficult when rat ES cells were cultured in N2B27-2i medium (Table 1).

Here, we successfully derived germline competent rat ES cells from DA rat. A series of identification in molecular and developmental levels have demonstrated that the rat ES cells are pluripotent, which expressed pluripotency makers and could contribute to the germline. It would accelerate the research in fundamental development processes and the underlying mechanisms of pathology by using rat ES cells and ES cell-based disease models, since rats would be better mimic the human disease than mouse.

In addition, we identified an optimized cultured medium N2B27-4i for rat ES cells, which not only promoted the proliferation but also improved stabilization of rat ES cells, especially during long-time culture. This suggested that the two additional inhibitors, ROCK inhibitor Y-27632 and TGF-β inhibitor A-83-01, might play important roles in the culture system of rat ES cells. Previous reports have demonstrated that Y-27632 was a potent inhibitor of apoptosis and permitted the survival of dissociated human ES cells and mouse ES cells during long-time culture (Watanabe et al., 2007); while A-83-01, the inhibitor of the type I TGF-β receptor ALK5, could improve the efficiency of induced pluripotent stem cells and stabilize the rat iPSC in culture (Li et al., 2009). Therefore, A-83-01 may contribute to maintaining the pluripotency of rat ES cells through inhibition of TGF-β signaling. However, it still needs further investigation in the future.

In summary, we successfully derived the first germline competent rat ES cells in China and found that the serum-free N2B27-4i medium was an optimized medium for rat ES cells culture. The derivation and availability of rat ES cells could accelerate the application of gene targeting and related disease models in rats.

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SUPPLEMENTARY DATA

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