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Establishment of rat embryonic stem-like cells from the morula using a combination of feeder layers

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Summary

Embryonic stem (ES) cells are characterized by pluripotency, in particular the ability to form a germline on injection into blastocysts. Despite numerous attempts, ES cell lines derived from rat embryos have not yet been established. The reason for this is unclear, although certain intrinsic biological differences among species and/or strains have been reported. Herein, using Wistar-Imamichi rats, specific characteristics of preimplantation embryos are described. At the blastocyst stage, Oct4 (also called Pou5f1) was expressed in both the inner cell mass (ICM) and the trophectoderm (TE), whereas expression of Cdx2 was localized to the TE. In contrast, at an earlier stage, expression of Oct4 was detected in all the nuclei in the morula. These stages were examined using a combination of feeder layers (rat embryonic fibroblast [REF] for primary outgrowth and SIM mouse embryo-derived thioguanine- and ouabain-resistant [STO] cells for passaging) to establish rat ES-like cell lines. The rat ES-like cell lines obtained from the morula maintained expression of Oct4 over long-term culture, whereas cell lines derived from blastocysts lost pluripotency during early passage. The morula-derived ES-like cell lines showed Oct4 expression in a long-term culture, even after cryogenic preservation, thawing and EGFP transfection. These results indicate that rat ES-like cell lines with long-term Oct4 expression can be established from the morula of Wistar-Imamichi rats using a combination of feeder layers.

Keywords: Cdx2, ES-like cells, Morula, Oct4, Rat

Introduction

In biology, the rat is an important model for studies in physiology, pathobiology, toxicology, neurobiology and a variety of other disciplines. The value of the rat as a biological model has led to an intense effort to establish it as a robust genetic model. However, key genetic technologies, such as targeted mutation of specific genes, are not available for the rat due to the inability to produce functional rat embryonic stem (ES) cells (Schulze *et al.*, 2006). In the mouse, ES cells represent one of the most important tools for genetic research and one of their primary applications is targeted mutation of specific genes by homologous recombination. In addition, ES cells are characterized by pluripotency, expression of the POU-domain germline-specific transcription factor Oct4 (also called Pou5f1) (Scholer *et al.*, 1989), germline formation upon injection into early embryos (Bradley *et al.*, 1984) and their capacity for somatic cell differentiation. Although there have been several attempts to isolate and culture pluripotent ES cell lines

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from different species, only ES cells from the mouse and chicken have been found to have germline competence (Prelle *et al.*, 1999).

In the rat, no ES cell line has been established despite numerous attempts (Iannaccone *et al.*, 1994; Brenin *et al.*, 1997; Vassilieva *et al.*, 2000; Buehr *et al.*, 2003; Ruhnke *et al.*, 2003). Indeed, even the formation of fully characterized cells with respect to the expression of ES cell markers has not been accomplished. It has been reported that a rat ES-like cell line expressing alkaline phosphatase (AP) and SSEA-1 was able to differentiate into neuronal, endothelial and hepatic lineages, although the cell line was immunonegative for Oct4 (Ruhnke *et al.*, 2003). In the rat, Oct4 has previously been rapidly lost in primary outgrowths of the majority of cultured embryos prior to any evidence of morphological differentiation (Buehr *et al.*, 2003). Therefore, it has been suggested that the lack of pluripotency of the rat embryonic cell lines could be due to Oct4 negativity. In establishing a rat ES cell line, a cell line maintaining Oct4 expression over long-term culture would be a significant step forward.

Previous studies have highlighted the complexity of the environment provided by the feeder cells and some of the basic differences in self-renewal mechanisms and pluripotency between mouse and human ES cells (Mannello & Tonti, 2007). Although differences in rat embryos and their derivatives compared with the mouse have been indicated, basic characteristics of pluripotency in rat embryos during preimplantation development and their compatibility with supportive feeder cells to establish ES-like cells are still unclear. Furthermore, the strain would be also an important factor, because the mouse strain is associated with the success of ES cell establishment. In the rat, strain-dependent efficiency of derivation of ES-like cells has also been noted (Vassilieva *et al.*, 2000; Fandrich *et al.*, 2002; Demers *et al.*, 2007). Previous studies have examined several strains, such as ACI, BD-IX, Brown Norway (BN), Fisher (F344), PVG, Sprague-Dawley (SD), Wistar-Kyoto and Wistar-Schönwalde; however, no rat ES cells have been obtained (Iannaccone *et al.*, 1994; Brenin *et al.*, 1997; Vassilieva *et al.*, 2000; Fandrich *et al.*, 2002; Buehr *et al.*, 2003; Ruhnke *et al.*, 2003; Demers *et al.*, 2007). Therefore, other strains need to be examined to determine whether a rat ES cell line can be established.

In the present study, using Wistar-Imamichi rats, the distribution of Oct4 in rat embryos during preimplantation and a combination of feeder layers was evaluated to establish rat cell lines that exhibit expression of ES cell markers. The present results indicated that Oct4 was expressed in the trophectoderm (TE). Therefore, the morula was used to establish ES-like cells. After EGFP transfection, expression of Oct4 was detected in EGFP-positive ES-like cells in a long-term culture. These findings suggest that the morula, along with a combination of feeder layers, can be used to establish rat ES-like cells, although further improvement is still required.

Materials and methods

Animals

Wistar-Imamichi rats were purchased from Japan SLC and bred in our animal care facility. Lights were on between 5:00 and 19:00 daily. At proestrus, females were caged with males for mating and the presence of vaginal plugs and/or sperm in the vaginal smear the next morning was assumed to indicate the first day of pregnancy (day 1). While conducting the research described in this study, the investigators adhered to the Guide for the Care and Use of Laboratory Animals published by Utsunomiya University, Utsunomiya, Japan.

Immunofluorescence detection of Oct4 and Cdx2 in preimplantation embryos

To localize Oct4 and Cdx2 in the embryos, immunohistochemical analysis was performed as described previously with slight modifications (Matsumoto *et al.*, 1998b, 2004). Briefly, embryos at the 2-cell, 4-cell, morula and blastocyst stages were collected on days 2 (14:00), 3 (12:00), 4 (18:00) and 5 (10:00), respectively. The embryos were fixed with 3.7% formaldehyde and permeabilized in 2.5% Tween 20, then incubated sequentially with goat anti-Oct4 polyclonal antibody (Santa Cruz Biotechnology, Inc.), Alexa594-conjugated donkey anti-goat antibody (Zymed Laboratories), mouse anti-Cdx2 monoclonal antibody (BioGenex) and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody (MP Biomedicals). Nuclei were labelled with 5 μg/ml Hoechst 33342. Immunodetection of Oct4 was performed for blastocysts injected with *EGFP* transgene-MoRE21 cells. The embryos were mounted on glass-based dishes (Asahi Glass Co.) and viewed using an Axiovert 200MOT-LSM (Carl Zeiss Inc.). Images shown in the results section are representative of at least 30 embryos from four to six different animals.

Derivation and culture of rat ES-like cell lines

Derivation of rat ES-like cell lines was carried out according to the procedures used for mouse ES cell line derivation or rat ES-like cells as described previously (Hogan *et al.*, 1994; Fandrich *et al.*, 2002; Buehr *et al.*, 2003). The stem cell medium (SCM) was composed of high-glucose Dulbecco's modified Eagle's medium, 20% heat-inactivated fetal bovine serum,

4 mM L-glutamine, nucleosides (8.0 μg/ml adenosine, 8.5 μg/ml guanosine, 7.3 μg/ml cytidine, 7.3 μg/ml uridine and 2.4 μg/ml thymidine), non-essential amino acids, 0.1 mM β-mercaptoethanol and antibiotic–antimicotic solution (all obtained from Sigma-Aldrich Corp.). As the feeder layer, a mitomycin C-treated monolayer of rat embryonic fibroblast (REF) or SIM mouse embryo-derived thioguanine- and ouabain-resistant (STO) cells was used for both derivation and long-term culture. Blastocysts were collected, transferred onto the feeder layer in 96 wells and left unattended for 1–2 days in SCM. During this period, blastocysts hatched and attached to the feeder cell layer and the inner cell mass started growing. Embryos at the morula stage were collected, the zona pellucida was removed by treatment with acidic Tyrode's solution and the embryos were transferred to culture medium on a feeder layer. Cells derived from rat embryos were allowed to outgrow for 4–5 days and were then mechanically detached. These cells were disaggregated with trypsin/EDTA (0.25%/0.04%) and moved to fresh feeders in culture medium. The resulting outgrowths were passaged by trypsinization every 2 or 3 days.

Characterization and transfection of rat ES-like cells

Alkaline phosphatase (AP) staining and immunodetection for Oct4 and SSEA-1 were performed as described previously with slight modifications (Hogan *et al.*, 1994; Matsumoto *et al.*, 2002b). For immunodetection, cells fixed with 3.7% formaldehyde were incubated sequentially with goat anti-Oct4 polyclonal antibody (Santa Cruz Biotechnology, Inc.) or mouse anti-SSEA-1 monoclonal antibody (Santa Cruz Biotechnology, Inc.), biotinylated secondary IgG antibody and horseradish peroxidase-conjugated streptavidin (Zymed Laboratories). Reactions were visualized using 3-amino-9-ethyl carbazole (Zymed Laboratories) as a chromogen. The plasmid (pCX–EGFP) of the *EGFP* gene that contains the cytomegalovirus (CMV) enhancer, chicken β-actin promoter and the rabbit β-globin 3′-flanking sequence was subcloned (Okabe *et al.*, 1997). The MoRE21 cell line was cryopreserved at the eighth passage and EGFP gene transfection was performed at the seventh passage after thawing (the fifteenth passage in total). Transfection of the MoRE21 cell line using the SalI digested vector was carried out using Lipofectamine 2000 (Invitrogen) as described in the manufacturer's instructions. EGFP expression was evaluated using fluorescence microscopy and cells were disaggregated with trypsin/EDTA (0.25%/0.04%). EGFP-positive cells were collected by pipetting and moved to fresh feeders in culture medium. The EGFP-positive MoRE21cell line was passaged by trypsinization every 2 or 3 days. The karyotype for the MoRE21 cell line at passage 15 after *EGFP* transgene was analysed as described previously with slight modifications (Kageyama *et al.*, 2004; Ulloa Ulloa *et al.*, 2008).

Blastocyst injection, embryo transfer and *in vitro* culture

Micromanipulation procedures were performed as described previously with slight modifications (Shinozawa *et al.*, 2006). For blastocyst injection, ES-like cells were trypsinized, resuspended in SCM and pre-plated on a tissue culture dish for 1 h to remove feeder cells and debris. The manipulation medium was SCM, identical to that used for the rat ES-like cell lines. Rat host blastocysts were injected with approximately 10 MoRE21 cells each using a flat-tip microinjection pipette with an internal diameter of 15–20 μm fitted to a piezo micromanipulator (Primetech). Blastocysts were transferred to the uteri of pseudopregnant recipients on day 5 as described previously (Shinozawa *et al.*, 2006). To promote Oct4 expression in MoRE21 cells injected into blastocysts, culture conditions were examined. Blastocysts containing injected cells were cultured in RaM (the previously described medium for rats) (Matsumoto & Sugawara, 1998; Matsumoto *et al.*, 1998a, 2002a), SCM, or conditioned SCM medium prepared from the supernatant of the ES-like cells with feeder layer culture.

Statistical analysis

Chi-squared analysis was used to evaluate statistical differences. A p-value less than 0.05 was considered significant. Comparisons with expected values of less than 5 were analysed using Fischer's Exact probability test.

Results

Distributions of Oct4 and Cdx2 in rat embryos during preimplantation development

The distributions of Oct4 and Cdx2, associated with pluripotency and lineage differentiation, were first examined. While neither Oct4 nor Cdx2 was expressed in nuclei at the 2-cell stage, nuclear expression of Oct4 was detected at the 4-cell stage (Fig. 1*a*, *b*). At the morula stage, both Oct4 and Cdx2 were expressed in all nuclei (Fig. 1*c*). Interestingly, at the blastocyst stage, Oct4 was expressed in both the ICM and TE, whereas expression of Cdx2 was localized only to the TE (Fig. 1*d*, *e*).

Appropriate feeder layer combination to establish rat ES-like cells

Feeder layer-dependent efficiency of cell line derivation for the rat was observed when standard mouse ES cell derivation protocols were performed on rat blastocysts.

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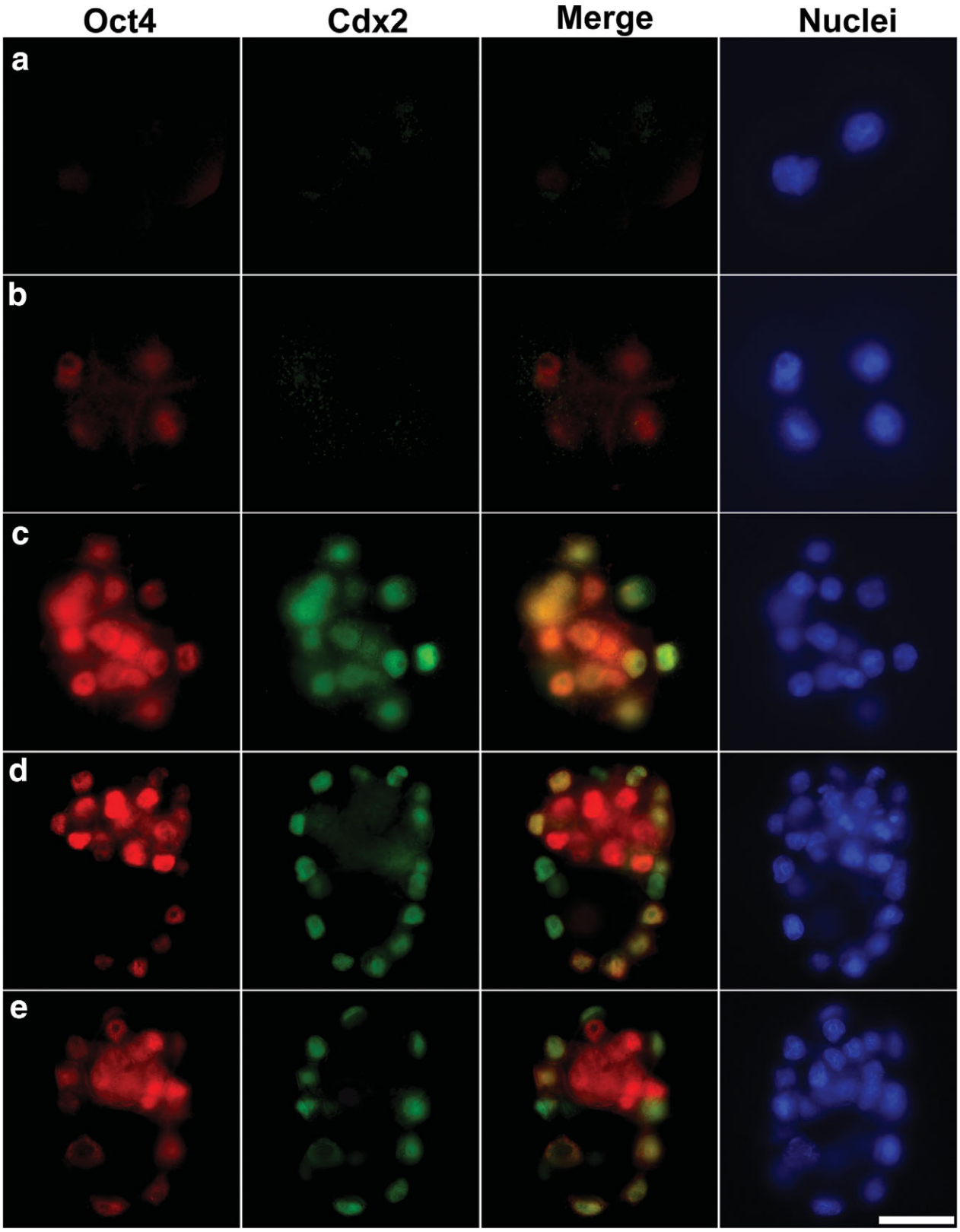

Figure 1 Oct4 and Cdx2 expression in rat embryos during preimplantation development. (*a*) 2-cell stage. (*b*) 4-cell stage. (*c*) Morula. (*d*, *e*) Blastocysts. Bar, 30 μm.

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Table 1 Dependence of the efficiency of rat ES-like cell line derivation on the combination of feeder layers.

Feeder layers for		No. of attached blastocysts	No. (%) of outgrowths	No. of AP-positive cell lines at passage				
Primary	Passage			3	4	5	6	7
STO	STO	29	20 (69.0)[a]	4	3	0	0	0
REF	REF	24	22 (91.7)[a,b]	5	0	0	0	0
REF	STO	38	36 (94.7)[b]	11	6	2	1	1

[a,b]Values with different superscripts within each column are significantly different ($p < 0.05$).

Table 2 Stage-dependent efficiency of rat ES-like cell line derivation.

Stage of embryo	No. of attached embryos	No. (%) of outgrowths	No. (%)* of AP-positive cell lines at the 7th passage
Blastocyst	63	54 (85.7)[a]	0 (0.0)[a]
Morula	75	57 (76.0)[a]	6 (10.5)[b]

[a,b]Values with different superscripts within each column are significantly different ($p < 0.05$).
*Based on the number of outgrowths.

As shown in Table 1, REF was effective as the feeder layer for primary outgrowth and derivation of ES-like cells, while STO increased the number of passages for cell lines. Although the combination of feeder layers improved rat ES-like cell derivation as judged by positive AP activity, only one cell line was AP-positive at the seventh passage.

Expression of ES cell markers by rat morula-derived cell lines

Although a combination of feeder layers was effective for derivation of rat ES-like cells from blastocysts, their AP activities were lost at early passage. Therefore, derivation of cell lines from the morula was examined with a combination of feeder layers, REF for primary outgrowth and STO for passaging, to establish rat ES-like cells. As shown in Table 2, the number of cell lines possessing AP activity at the seventh passage was increased. The morphology of these cell lines, which are large aggregates of cuboidal or cobblestone-shaped cells (Fig. 2*b*), was similar to that described previously for the rat (Demers *et al.*, 2007). These aggregates maintained a large size at the eighth passage, whereas the blastocyst-derived cell lines showed smaller aggregates at earlier passages (Fig. 2*a*, *b*). Other markers of undifferentiated cells, i.e., Oct4 (Fig. 2*c*) and SSEA-1 (Fig. 2*d*), were also found to be uniformly positive with regard to the AP-positive cell lines. At passage 7 or 8, these cell lines were cryopreserved.

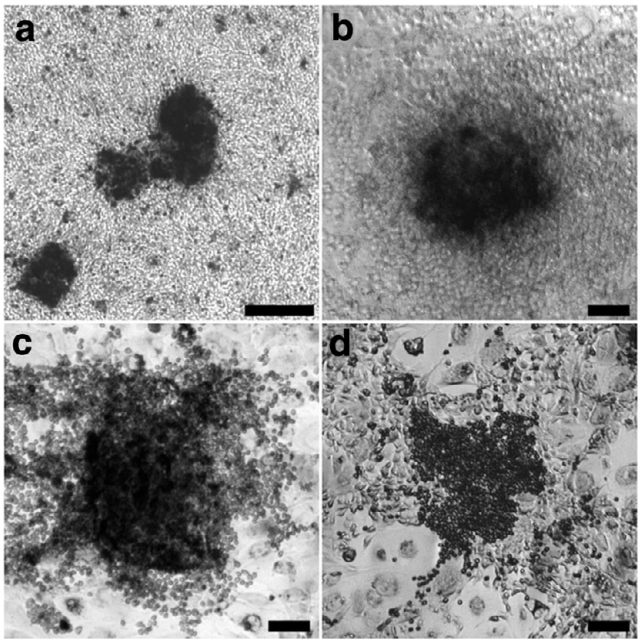

Figure 2 Rat embryo-derived cell lines exhibit morphological and molecular markers of undifferentiated cells. (*a*) Positive alkaline phosphatase (AP) staining of rat blastocyst-derived cells at passage 4. (*b*) Rat morula-derived cell lines with AP-positive staining at passage 8. (*c*) Oct4 and (*d*) SSEA-1 expression in rat morula-derived cell lines. Bars, 100 µm.

Characteristics of the EGFP transgene-expressing MoRE21 rat ES-like cell line

The cryopreserved MoRE21 rat ES-like cell line was thawed, followed by transfection with the pCX–EGFP vector. EGFP transgene expression in the transfected cells was confirmed by fluorescence microscopy and these cells were collected for passaging by pipetting. Karyotype analysis at passage 15 after EGFP transfection showed that 57.1% of 28 metaphase plates contained 42 chromosomes—the normal diploid complement for the rat (Fig. 3), similar to previous results (Brenin *et al.*, 1997; Demers *et al.*, 2007). The

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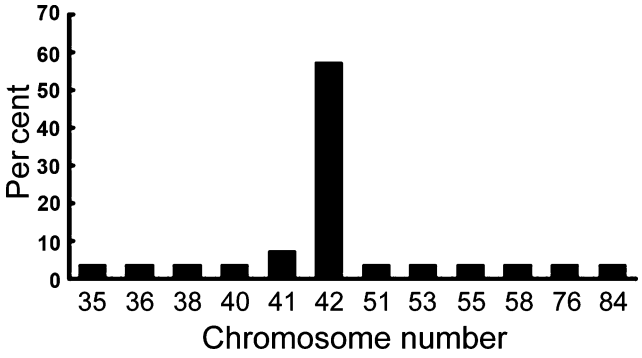

Figure 3 Distribution of chromosomes in *EGFP* transgene MoRE21 rat ES-like cell line.

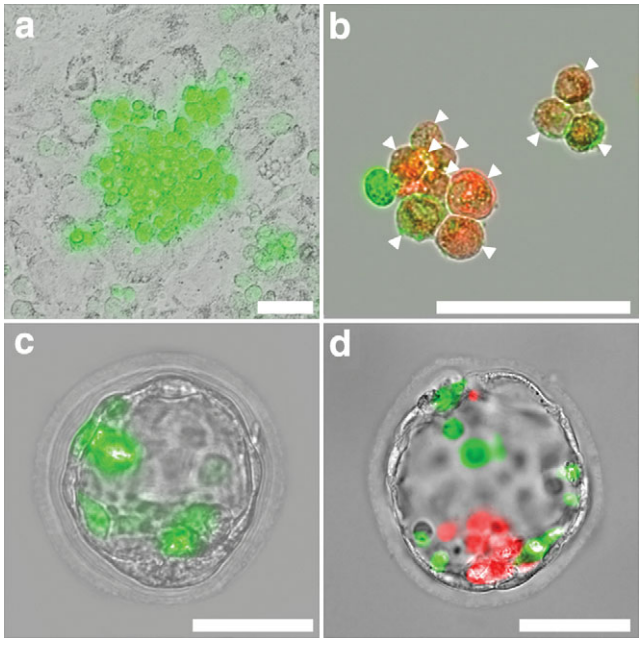

Figure 4 Distributions of EGFP and Oct4. (*a*) *EGFP* transgene expression in the MoRE21 rat ES-like cell line. (*b*) Immunohistochemical detection of Oct4 (red) in EGFP-positive MoRE21 cells before injection into blastocysts. The arrowheads indicate Oct4 expression in EGFP-positive cells. (*c*) Localization of injected EGFP-expressing MoRE21 cells in a live blastocyst after 24 h of *in vitro* culture. (*d*) No Oct4 expression was detected in EGFP-positive cells injected into a cultured blastocyst, while Oct4 was detected in the ICM. Bars, 50 μm.

EGFP-positive MoRE21cell line was also positive until passage 90 after EGFP transfection (Fig. 4*a*). The number of Oct4 positive cells was counted using immunofluorescence analysis for both low (twenty third) and high (forty sixth) passage numbers after EGFP transfection. On repeating the staining at higher passage numbers, no obvious changes were noted. Oct4 expression among EGFP-positive MoRE21 cells was 69.1% (1462/2115).

Potential of rat ES-like cells injected into blastocysts

To address the developmental capacity and pluripotency of the MoRE21 rat ES-like cells, they were injected into blastocysts to investigate the contribution of this cell line to embryos. Approximately 10 EGFP-positive MoRE21 cells were injected into each host blastocyst. A total of 32 injected blastocysts were transferred into five pseudopregnant recipients and pregnancy was examined. Although all of the recipients showed decidual swellings (78.1%, 25/32), only one live birth by Caesarean section occurred; EGFP expression was not detected (data not shown). Therefore, the expression of Oct4 in injected rat ES-like cells in blastocysts was examined using immunohistochemical analysis. As shown in Fig. 4*b*, Oct4 was detected in EGFP-positive MoRE21 cells before injection into the blastocysts. Although EGFP-expressing cells and blastocysts were revealed to have a normal morphology (Fig. 4*c*), Oct4 expression disappeared in the EGFP-positive cells after 24 h culture in RaM ($n = 10$) (Fig. 4*d*). Furthermore, no embryos expressed Oct4 in the injected cells after 24 h culture in SCM ($n = 11$) or conditioned SCM medium ($n = 18$).

Discussion

In the rat, none of the published cell lines have proven to be genuine ES cells, as defined by their contribution to the germline. Although the reason it is difficult to establish rat ES cells is unclear, it has been supposed that the presence of certain intrinsic biological differences between rats and mice accounts for the species-to-species variation in the derivation of ES cells. For example, the rat epiblast retains its ability to generate parietal endoderm cells *in vitro*, whereas the mouse epiblast no longer possesses this capacity (Nichols *et al.*, 1998a). When isolated rat epiblasts are transplanted under the kidney capsule of isogenic adult males, isografts result in the formation of benign teratomas after 4 weeks and developed into malignant yolk sac carcinomas after longer periods (Knezevic *et al.*, 2005). Furthermore, mouse teratocarcinomas are germ cell tumours, whereas rat yolk sac carcinomas are of extraembryonic origin (Sobis *et al.*, 1993; Brenin *et al.*, 1997). Therefore, embryonic differences between rats and mice may be associated with the difference in pluripotency in cell lines derived from embryos.

In preimplantation embryos, Oct4 is restricted to the ICM, whereas Cdx2 is localized to the TE in mouse blastocysts. Oct4 is essential to the pluripotent character of the mouse ICM and derivative ES cells (Nichols *et al.*, 1998b). Because Oct4-mutant blastocysts contain only the TE, ICM formation depends on Oct4. In contrast, Cdx2, a caudal-type homeodomain protein,

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is crucial for segregating ICM and TE lineages at the blastocyst stage by ensuring the repression of Oct4 in the TE (Strumpf *et al.*, 2005). In contrast to the mouse, the present results showed that Oct4 was expressed in both the ICM and TE in rat blastocysts, whereas expression of Cdx2 was localized only to the TE. Similar results have been obtained for other species, i.e., porcine (Kirchhof *et al.*, 2000), bovine (van Eijk *et al.*, 1999; Kirchhof *et al.*, 2000) and human (Cauffman *et al.*, 2005; Cauffman *et al.*, 2006). In mammals, only ES cells from the mouse have been obtained that have germline competence. Therefore, the different Oct4 expression in blastocysts could be related to subsequent distinctions in differentiation between rats and mice, although the mechanism of differential distribution is unclear.

The morula is a stage of differentiation prior to the blastocyst that consists of ICM and TE. The present results indicate that use of the morula made possible establishment of an Oct4-expressing rat ES-like cell line over long-term culture. This is the first attempt to examine the rat morula stage for potential development of ES-like cells, although it has been reported that ES cells can also be derived from the morula stage in the mouse (Tesar, 2005). Furthermore, a combination of feeder layers was examined. Previous studies on the rat used feeder layers derived from rat (Iannaccone *et al.*, 1994; Brenin *et al.*, 1997), mouse (Vassilieva *et al.*, 2000; Ruhnke *et al.*, 2003; Demers *et al.*, 2007) and transfected mouse cells (Buehr *et al.*, 2003). In contrast, the present combination of feeder layers improved the ability to obtain a rat ES-like cell line. These results suggest that different cell types are compatible during primary outgrowth and passaging for rat embryos.

The present results indicate that rat ES-like cell lines expressing Oct4 over long-term culture could be obtained from the morula, whereas cell lines derived from blastocysts lost AP activity during early passage. Previously, a rat AP-positive ES-like cell line was obtained from Wistar-Kyoto blastocysts (Fandrich *et al.*, 2002), whereas Wistar-Imamichi rats were used in this study. The strain is an important factor in establishing mouse ES cell lines. Therefore, the lack of AP-positive cells from Wistar-Imamichi blastocysts may be a strain-dependent trait. In addition, use of morulae from other strains may allow establishment of rat ES-like cells with higher pluripotency.

For rat ES-like cells, it has been reported that dissociation with enzymatic buffers during early subcultures resulted in immediate differentiation, while clonal assays used only enzymatic dissociation because mechanical disaggregation did not produce single-cell suspensions (Vassilieva *et al.*, 2000). Recently, using mechanical passaging, one rat ES-like cell line that maintained Oct4 expression in a long-term culture was established from blastocysts (Demers *et al.*, 2007). This cell line was capable of contributing to chimeras as extraembryonic tissues, but not to germline or to embryonic tissues. In the present study, both blastocyst- and morula-derived cells were passaged by trypsinization and the present results showed that an AP-positive cell line was not obtained from blastocysts, whereas morula-derived rat ES-like cell lines maintained Oct4 expression over long-term culture. Therefore, rat ES-like cells derived from the morula may be resistant to enzymatic dissociation compared to those derived from blastocysts.

In the present study, the ES-like cell lines derived from the morula maintained Oct4 expression over long-term culture, even after cryopreservation, thawing and EGFP transfection. However, Oct4 expression in the cells injected into the blastocysts disappeared within 24 h of culture. This rapid loss of Oct4 was not prevented by any culture medium, even the conditioned culture medium from ES-like cells with a feeder layer. Therefore, pluripotency in these rat ES-like cells may be unstable in blastocysts post-injection. Attachment with feeder layer could be necessary to maintain Oct4 expression in these rat ES-like cells. Because strain-dependent efficiency of rat ES-like cell derivation has been reported (Vassilieva *et al.*, 2000; Fandrich *et al.*, 2002; Demers *et al.*, 2007), morula-stage embryos derived from other strains with a combination of feeder layers may allow establishment of ES-like cell lines that can contribute to embryonic tissues. Our results indicated the characteristics of preimplantation embryos and their derivations in rat. Further research for the differentiation during compaction and blastulation in rat embryos could provide useful information to establish rat ES cells.

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Addendum

During preparation of this manuscript, Li and colleagues reported the blastocyst-derived rat ES cells.

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