Int. J. Dev. Biol. 57: 845-852 (2013)
doi: 10.1387/ijdb.130128sl

THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY
www.intjdevbiol.com

The *Oct4* promoter-*EGFP* transgenic rabbit: a new model for monitoring the pluripotency of rabbit stem cells

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ABSTRACT The rabbit has long been used as a laboratory animal model for developing reproductive and stem cell-related technologies, as well as for studying human disease. The Oct4 transcription factor plays a crucial role in the maintenance and regulation of pluripotency in embryos and stem cells. We constructed a reporter plasmid containing the gene encoding the enhanced green fluorescent protein (EGFP) under the control of the rabbit *Oct4* promoter (prOG) and transfected it into E14 mouse stem cells and rabbit ESCs. In addition, prOG transgenic fibroblasts were derived and prOG transgenic rabbits were produced by somatic cell nuclear transfer (SCNT). The pattern of expression of ectopic EGFP was similar in E14 mouse stem cells whether under the control of the rabbit (prOG) or mouse Oct4 promoter (pmOG). *EGFP* expression was observed in rabbit ESCs following prOG transfection. Both prOG transgenic SCNT embryos and F1 prOG transgenic embryos derived from adult transgenic rabbits expressed green fluorescence at the morula and blastocyst stages. EGFP was clearly detected in gonads isolated from fetuses at 27 dpc. The prOG transgenic rabbit represents a new model for studying the derivation and maintenance of rabbit pluripotent cells, and for investigating rabbit embryo development.

KEY WORDS: *Oct4, rabbit, stem cell, EGFP, development*

Introduction

Embryonic stem (ES) cells are capable of self-renewal and differentiation into many different cells and tissues comprising all three germ layers, both *in vitro* and *in vivo*. Therefore, ES cells are important for research into developmental biology, cell-based therapies, and genetic engineering (Do and Scholer, 2009). Since the first successful murine experiments (Evans and Kaufman, 1981, Martin, 1981), ES cell lines have been successfully established in a number of species, including humans, monkeys, and rats (Thomson *et al.*, 1998). Recently, a new method was established for producing induced pluripotent stem (iPS) cells from mouse somatic cells by overexpressing four key transcription factors (Takahashi and Yamanaka, 2006). Within a year, human iPS cell lines were created using the same technology (Takahashi *et al.*, 2007). Now iPS cells have been successfully produced from many other species (Ezashi *et al.*, 2009, Honda *et al.*, 2010, Liu *et al.*, 2008, Sumer *et al.*, 2010). Further improvements in ES and iPS cell techniques will enable cell-based therapies to be developed (Grigoriadis *et al.*, 2010, Zou *et al.*, 2011). Animal models will play important roles in the preliminary testing of novel ES or iPS cell-based therapies, which is required for ethical approval to be granted for clinical trials (Okamoto and Takahashi, 2011, Zou *et al.*, 2011). Rabbits have several attractive features of small animal models, such as easy housing and handling. In addition, rabbits are preferentially used in cardiovascular, metabolic, myocardial, and pulmonary studies (Largo *et al.*, 2008, Shimizu *et al.*, 2009). While rabbit ES-like cells share many characteristics of true ES cells (Wang *et al.*, 2007) and rabbit iPS cells have been generated (Honda *et al.*, 2010, Osteil *et al.*, 2013), more research into establishing rabbit ES cell lines is required.

Oct4, a member of the POU (Pit-Oct-Unc) family of transcription factors, is widely used as a pluripotent cell marker in many species (Wuensch *et al.*, 2007, Zuccotti *et al.*, 2012). Oct4 plays a crucial role in transcriptional regulation during early embryonic development and in cell differentiation (Kellner and Kikyo, 2010,

Abbreviations used in this paper: BSA, bovine serum albumin; dpc, days postcoitum; EGFP, enhanced green fluorescent protein; ES, embryonic stem cell; FBS, fetal bovine serum; ICM, inner cell mass; iPS, induced pluripotent stem cell; PBS, phosphate-buffered saline; PGCs, primordial germ cells; RT, room temperature; SCNT, somatic cell nuclear transfer; ZGA, zygotic genomic activation.

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Accepted: 10 September 2013. *Final, author-corrected PDF published online:* 20 January 2014. *Edited by:* Sally Dunwoodie.

ISSN: Online 1696-3547, Print 0214-6282

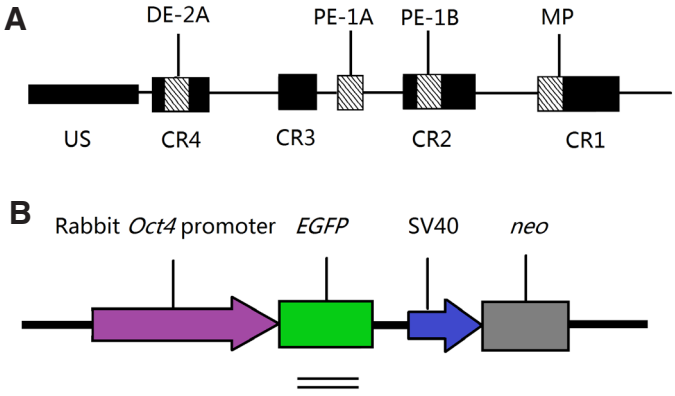

Fig. 1. Diagram of the rabbit Oct4 promoter and the prOG vector. (A) *Diagram of the rabbit* Oct4 *upstream promoter region (approximately 3.2 kb; black bars represent conserved regions). There are four main elements: the distal enhancer (DE), the proximal enhancer (PE), and the minimal promoter (MP) and part of the upstream sequence (US).* **(B)** *Diagram of the prOG vector, containing the rabbit* Oct4 *promoter (approximately 3.2 kb) followed by the* EGFP *gene (approximately 1 kb) and a neomycin resistance gene (*neo*). The* EGFP *probe used for Southern blotting is indicated by double lines.*

et al., 1998) and with murine PGC apoptosis (Kehler *et al.*, 2004), suggesting an important role for Oct4 in the maintenance and self-renewal of pluripotent cell populations (Boiani and Scholer, 2005). Therefore, *Oct4* is a good candidate gene for directing reporter gene expression in embryonic and pluripotent stem cells. The Oct4-enhanced green fluorescent protein (EGFP) mouse has revealed the timing and tissue specificity of *Oct4* expression, and has been extremely valuable model for basic studies on pluripotency and differentiation (Yoshimizu *et al.*, 1999). Subsequently, *Oct4–EGFP* reporter systems have been widely used for investigating the pluripotency and the reprogramming process in many other species, including human, mouse, pig and cattle (Berg *et al.*, 2011, Nowak-Imialek *et al.*, 2011, Ruiz *et al.*, 2012, Scaldaferri *et al.*, 2011, Youn *et al.*, 2013).

The rabbit *Oct4* cDNA and genomic DNA sequences are available and gene expression in preimplantation embryos has been characterized (Mamo *et al.*, 2008, Shi *et al.*, 2008). Moreover, the rabbit *Oct4* 5' regulatory region was sequenced and phylogenetically conserved regions were identified (Kobolak *et al.*, 2009).

In this study, we constructed a prOG vector in which the rabbit *Oct4* promoter controls expression of the gene encoding EGFP. The prOG transgenic embryos and rabbits were produced by somatic cell nuclear transfer (SCNT). Both prOG SCNT embryos and prOG F1 embryos derived from a transgenic rabbit expressed EGFP. In addition, green fluorescence was readily detected in gonads isolated from 27-dpc fetuses. We expect the prOG transgenic rabbit to be a useful animal model for studying the isolation and maintenance of rabbit pluripotent cells, and for conducting research into rabbit embryo development.

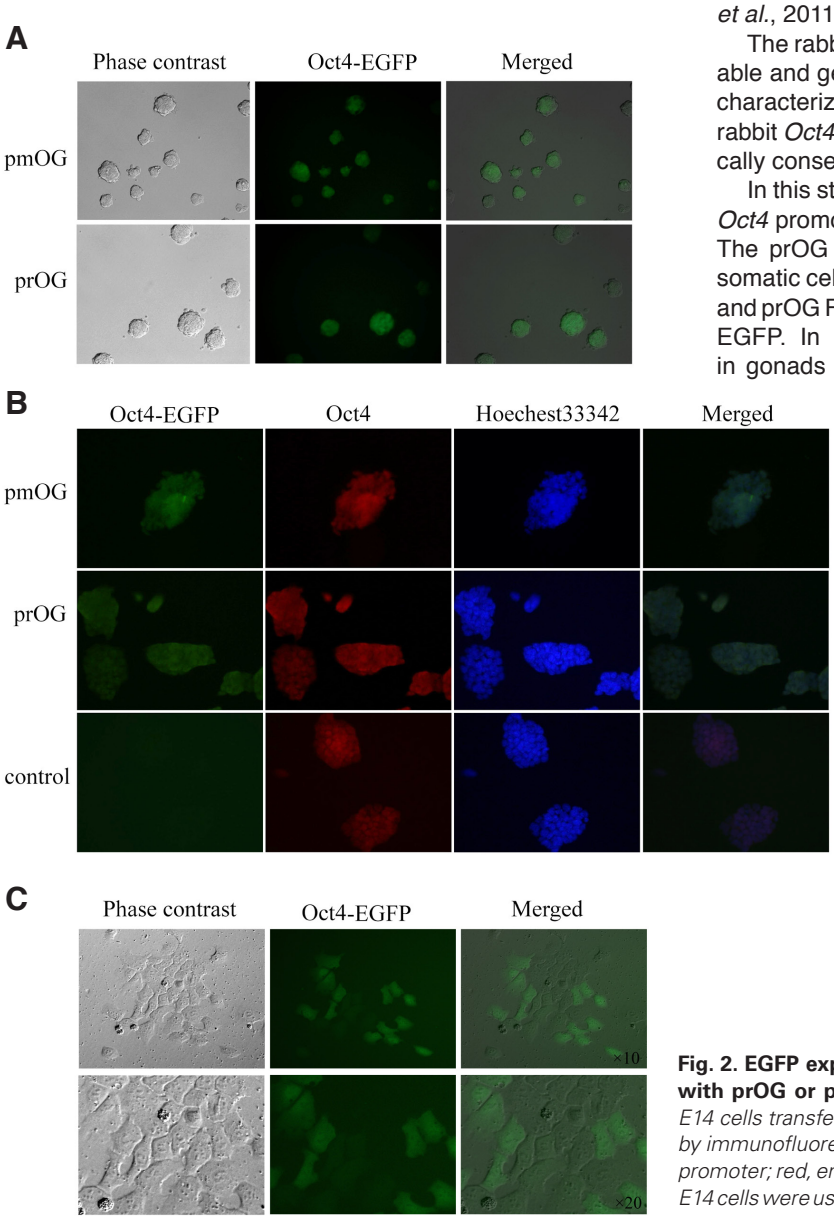

Fig. 2. EGFP expression in mouse E14 cells and rabbit ESCs transfected with prOG or pmOG reporters. (A) *EGFP fluorescence (EGFP) images of E14 cells transfected with pmOG and prOG.* **(B)** Oct4 *expression is assessed by immunofluorescence. Green, ectopic* EGFP *expression driven by the* Oct4 *promoter; red, endogenous Oct4; blue, Hoechst33342 staining. Untransfected E14 cells were used as controls.* **(C)** *Rabbit ESCs transfected with prOG reporter.*

Results

prOG and pmOG transfection induces similar pattern of EGFP expression

A 3.2 kb fragment of the *Oct4* upstream promoter region was amplified from rabbit genomic DNA. Part of this sequence is highly homologous to rabbit *Oct4* promoter regions identified by BLAST analysis (Kobolak *et al.*, 2009) and contains four extensively conserved regions (CR1–4) and three main elements, the distal enhancer (DE), the proximal enhancer (PE), and the minimal promoter (MP). Another approximately 1 kb sequence upstream of the *Oct4* promoter region was named US (upstream; Fig. 1A). In addition, the mouse *Oct4* promoter was amplified from the mouse genome. These promoter fragments were used to construct prOG

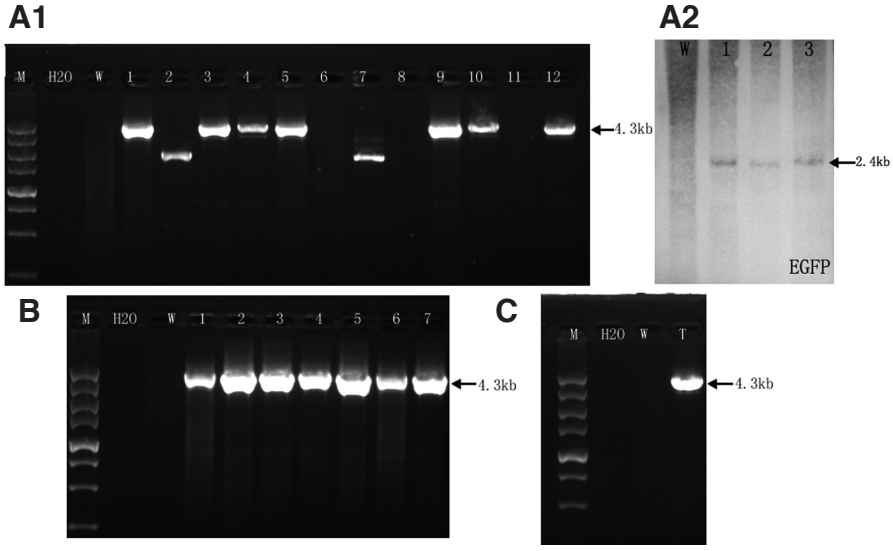

Fig. 3. PCR detection of Oct4 promoter–EGFP transgene. (A1) *PCR analysis of DNA isolated from G418-resistant rabbit fetal fibroblasts stably transfected with the* Oct4 *promoter–*EGFP *transgene. M, DL5000 marker; W, wild-type rabbit fibroblasts (negative control); Lanes 1–12, G418-resistant rabbit cell clones. Positive clones were lines 1, 3, 4, 5, 9, 10, and 12 (indicated by band of approximately 4.3 kb).* **(A2)** *Southern blot analysis of genomic DNA from PCR-positive cell clones showing hybridization with the EGFP probe after* Xba*I digestion. W, wild-type rabbit (negative control); Lanes 1–3: transgenic cell line 1, 3, and 5. The arrow marks the labeled 2.4 kb fragment.* **(B)** *PCR analysis of DNA isolated from stillborn transgenic rabbits. M, DL5000 marker; W, wild-type rabbit; Lanes 1–7, Oct4–EGFP stillborn transgenic rabbits.* **(C)** *PCR analysis of the surviving transgenic rabbit. M, DL5000 marker; W, wild-type rabbit; T, Oct4–EGFP transgenic rabbit.*

(rabbit *Oct4* promoter-*EGFP*; Fig. 1B) and pmOG (mouse *Oct4* promoter-*EGFP*) vectors.

EGFP expression was observed in E14 mouse ES cells 2 days after transfection with either the prOG or pmOG vector. After G418 selection, stably transfected clones were mechanically isolated and subcultured. Green fluorescence was detected in E14 mouse ES cells transfected with either the prOG or pmOG vector (Fig. 2A). *Oct4* expression in *Oct4-EGFP* transgenic mouse ES cells was assessed by immunofluorescence (Fig. 2B). These data confirm that both reporter vectors function correctly and that prOG is a specific marker for *Oct4*-expressing cells.

prOG transfection induces EGFP expression in rabbit ESCs

The prOG vector was transfected into rabbit ESCs using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). *EGFP* expression was observed in rabbit ESCs 2 days after transfection (Fig. 2C). However, following G418 selection, stably transfected clones were not obtained owing to cell differentiation.

The prOG construct is stably integrated into rabbit fibroblasts

Next, the prOG vector was transfected into rabbit fibroblasts to assess *EGFP* expression in embryos and rabbits obtained following SCNT. As expected, no fluorescence was observed in prOG-transfected fibroblasts. A total of 12 stably transfected clonal cell lines were obtained following 7–10 days of G418 selection. DNA was isolated from all cell lines and transgene integration was assessed by PCR. Seven cell clones were found to contain the entire exogenous rabbit *Oct4* promoter–*EGFP* sequence (approximately 4.3 kb; Fig. 3 A1). PrOG integration was confirmed in three fast-growing transgenic cell lines (1, 3, and 5) by Southern blotting using an *EGFP* probe (about 412 bp). The labeled fragment was about 2.4 kb (Fig. 3 A2). These data demonstrate that the *EGFP* fragment from the prOG plasmid is integrated into rabbit genome.

EGFP is expressed in both SCNT embryos and prOG transgenic rabbits

Two fast-growing transgenic cell lines were used as donor cells for SCNT. The total cleavage rate was 69.7% in cell line 1 and 73.6% in cell line 5. Further, the total blastocyst rate was 23.7% in cell line 1 and 21.1% in cell line 5 (Table 1). Green fluorescence was clearly observed in all morulae and blastocysts from SCNT (Fig. 4). EGFP was detected in both the ICM and trophectoderm of blastocysts. Parthenogenetic blastocysts were used as controls.

Production of prOG transgenic rabbits

A total of 177 SCNT embryos derived from two transgenic cell lines were transferred to pseudopregnant rabbits. Of the eight recipient rabbits, three had successful pregnancies and eight kits were delivered. Of these, one transgenic male rabbit survived for at least 1 year (up to the time of manuscript preparation; Table 1). The prOG transgene was analyzed in seven stillborn rabbits (Fig. 3B) and in the surviving rabbit (Fig. 3C) by PCR.

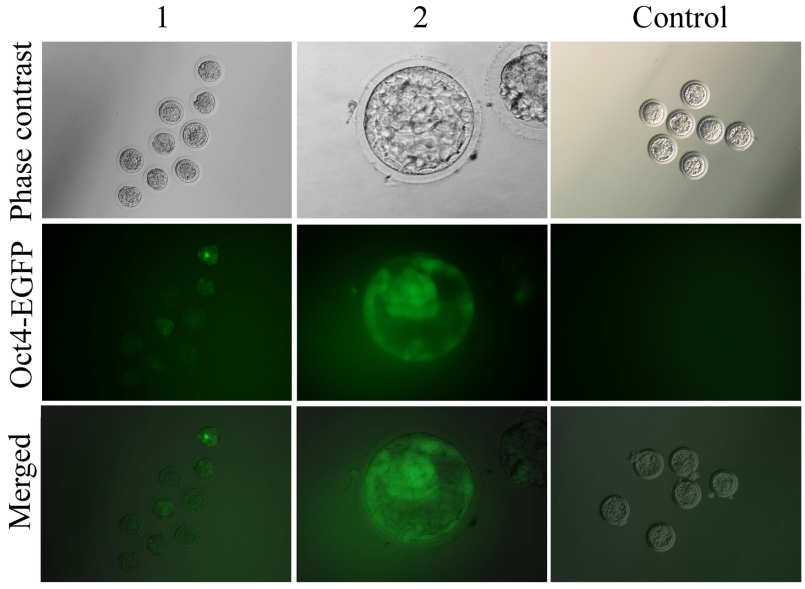

Fig. 4. *EGFP* expression in transgenic embryos. (1) *SCNT embryos reconstructed using transgenic cell lines.* **(2)** EGFP *expression in the inner cell mass and the trophectoderm at the blastocyst stage. Parthenogenetic rabbit embryos were used as controls.*

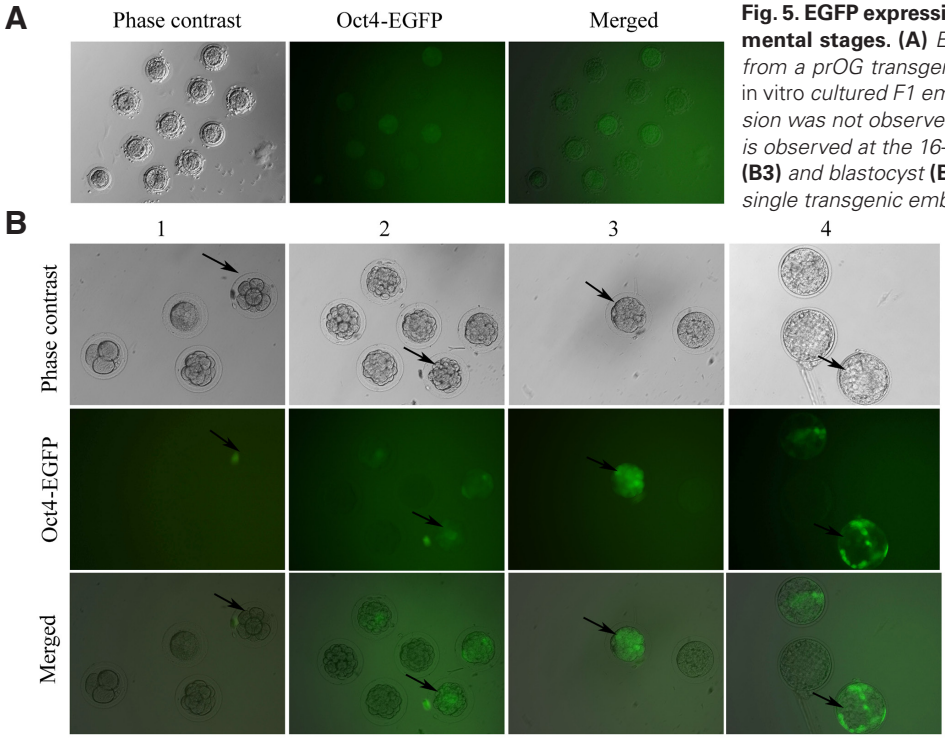

Fig. 5. EGFP expression in cultured F1 embryos at different developmental stages. (A) *EGFP expression in areas of cultured F1 embryos from a prOG transgenic rabbit.* **(B)** EGFP *expression in different stage* in vitro *cultured F1 embryos from prOG transgenic rabbit.* EGFP *expression was not observed prior to the 16-cell stage* **(B1)**. EGFP *expression is observed at the 16- to 32-cell stage* **(B2)** *and increases at the morula* **(B3)** *and blastocyst* **(B4)** *stages. (Arrows indicate* EGFP *expression in a single transgenic embryo at different stages)*

The Oct4-EGFP transgene is transmitted in the germ line

Germ line transmission was assessed by mating the transgenic rabbit with wild-type females. One day after mating, fertilized embryos were harvested and cultured in vitro. After two days, approximately 52.5% (31 of 59) of the F1 embryos expressed EGFP (Fig. 5A). Furthermore, stage-specific *EGFP* expression was observed in cultured F1 embryos derived from a prOG transgenic rabbit. *EGFP* expression was not observed before the 16-cell stage (Fig. 5 B1). At the 16- to 32-cell stage, *EGFP* expression is observed (Fig. 5 B2), which increases at the morula and blastocyst stages (Fig. 5 B3,4).

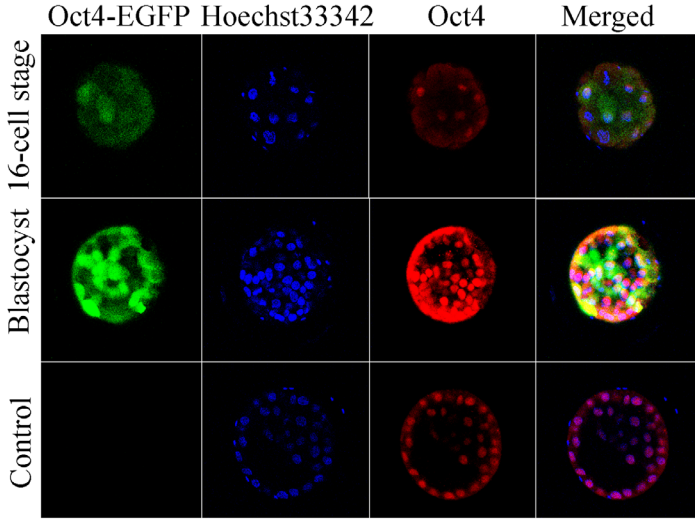

Fig. 6. *Oct-4* expression in prOG transgenic embryos. *All prOG embryos at the 16-cell or blastocyst stages expressing ectopic Oct4–EGFP (green) also expressed endogenous Oct4 (red). Nuclei are indicated by Hoechst33342 nuclear staining (blue). Normal blastocysts were used as control.*

Reporter expression is temporally and spatially similar to endogenous Oct4 expression

To determine whether *EGFP* expression driven by the rabbit *Oct4* promoter resembles endogenous *Oct4* gene expression, *EGFP*-expressing F1 embryos and wild-type controls at different developmental stages were assessed by immunofluorescence using an anti-Oct4 antibody. As expected, all prOG embryos expressed endogenous Oct4 at the 16-cell and blastocyst stages (Fig. 6). *Oct4-EGFP* reporter expression was similar to endogenous *Oct4* expression with respect to localization and developmental stage.

EGFP expression in the gonads of Oct4-EGFP transgenic fetuses

The germinal tissues of Oct4-EGFP transgenic fetuses at 15 dpc and 27 dpc were isolated and EGFP expression was analyzed. No green fluorescence was detected in the germinal ridges, which are connected with the mesonephros, but green fluorescence was detected in several cells between the two genital ridges in the fetus at 15 dpc (Fig. 7A). However, when the testes and ovaries isolated from fetuses at 27 dpc were analyzed, green fluorescence was clearly detected in the seminiferous tubules of the testes (Fig. 7B) and punctate green fluorescence was observed in the cortex of the ovaries (Fig. 7C). No green fluorescence was detected in any of the other tissues tested, including the heart, liver, and skin (data not shown). prOG transgene integration and sex were confirmed by PCR and morphological analysis, respectively. Transgene-negative gonads from 27-dpc fetuses were used as controls.

Discussion

In this study, an *Oct4-EGFP* reporter system was developed and the first transgenic *Oct4-EGFP* rabbit was made. The prOG transgenic rabbit is a useful tool for monitoring *Oct4* expression in embryonic and pluripotent stem cells. ES or iPS cells derived from the prOG rabbit will enable the mechanisms controlling rabbit pluripotent stem maintenance and reprogramming required for iPS production to be studied.

The upstream region of the rabbit *Oct4* gene was subcloned and four highly conserved promoter regions (CR1–4) were identified, as previously reported (Kobolak *et al.*, 2009). Sequence alignment of the mouse, human, and bovine *Oct4*

TABLE 1

SUMMARY OF NUCLEAR TRANSFER RESULTS USING PROG TRANSGENIC FIBROBLASTS

Donor cell lines	No. of reconstructed embryos	Cleavage (rate)	Blastocyst (rate)	No. of embryos transferred	No. of recipients recipients	No. of pregnant recipients	No. of live/ stillborn or postnatal deaths rabbits
Cell line 1	76	53 (69.7%)	18 (23.7%)				
Cell line 1	229	168 (73.3%)		134	6	3	1/7
Cell line 5	19	14 (73.6%)	4 (21.1%)				
Cell line 5	59	43 (72.8%)		43	2	0	0

upstream promoter sequences and pairwise comparisons of the rabbit *Oct4* regulatory regions with equivalent murine, bovine, canine, and human sequences revealed a high level of *Oct4* sequence conservation. The highest degree of sequence similarity was found in the conserved domains between rabbit and human. However, differences in some regions were found, especially in the PE region. A previous analysis of the rabbit *Oct4* promoter showed that different regions can induce different levels of reporter expression in mouse ES cells (Kobolak *et al*., 2009). The CR4 region is responsible for *Oct4* expression in ES cells (Yeom *et al*., 1996). To monitor *Oct4* expression in rabbit, we decided to express EGFP under the control of the entire rabbit *Oct4* promoter (approximately 3.2 kb, including approximately 1 kb more upstream sequence than the reported *Oct4* promoter region) to maximize reporter expression, increase the efficiency of exogenous transgenes, and maintain integrity of the CR4 region.

Well-established techniques are available for isolating and studying mouse ES cells. We therefore constructed the pmOG vector, containing the mouse *Oct4* promoter and *EGFP*, for comparing with the prOG vector. Both prOG and pmOG reporters were transfected into E14 mouse ES cells and the pattern of reporter expression was assessed by EGFP fluorescence. Both prOG and pmOG reporters were expressed at similar pattern in mouse ES cells. This study clearly shows the high degree of homology that exists between *Oct4* genes of different species and the similar level of activity of the rabbit and mouse promoter sequences (prOG and pmOG). In parallel experiments, rabbit ESCs were transfected with the prOG vector. The results confirm that *EGFP* is expressed in rabbit ESCs.

In an attempt to establish prOG transgenic rabbit fibroblasts, we chose two cell lines with intact *Oct4-EGFP* sequences for SCNT experiments. All transgenic SCNT embryos expressed EGFP and all prOG transgenic rabbits, both live and stillborn, contained the entire *Oct4* promoter–*EGFP* sequence. The results indicate that the rate of transgenic animal production can be improved by monoclonal selection of transfectants coupled with PCR and Southern blot confirmation of transgene integration.

SCNT and F1 blastocyst analysis revealed EGFP expression in both the ICM and trophectoderm (Fig. 4-2, Fig. 6), similar to previous reports of endogenous Oct4 mRNA expression (Kobolak *et al*., 2009). The pattern of *Oct4* expression in blastocysts differs among species. For example, Oct4 protein is detectable in the ICM but not the trophectoderm of *in vivo*-derived murine embryos; in contrast, Oct4 protein is expressed in both the ICM and the trophectoderm of *in vivo*- and *in vitro*-derived porcine and bovine blastocysts (Kirchhof *et al*., 2000). Previous studies have analyzed Oct4 expression in rabbit embryos using RT-PCR (Mamo *et al*., 2008) and immunostaining (Chen *et al*., 2012). Oct4 is expressed at the zygote stage, and expression decreases gradually until zygotic genomic activation (ZGA) (8–16-cell stage for rabbits) and then increases up to the blastocyst stage. In this study, *EGFP* expression was first observed at the 16- to 32-cell stage, and then increased up to the morula and blastocyst stages in F1 transgenic embryos (Fig.5B-2, 3, 4). This indicates that expression of the paternal *Oct4* gene commences with ZGA in rabbit embryos. The initial development of mammalian embryos is governed by gene transcripts and polypeptides produced by and stored in the oocytes during oogenesis (Schultz, 1993). Following cleavage, the amount of maternally derived transcripts and proteins gradually decreases (De Sousa *et al*., 1998) and transition from maternal to embryonic gene expression occurs. In rabbits, ZGA occurs at the 8–16-cell stage, more than 44 h after fertilization (Pacheco-Trigon *et al*., 2002). In the present study, a lack of *EGFP* expression before the 8-cell stage suggests that the integrated *Oct4-EGFP* sequence is not fully activated. Overall, *Oct4-EGFP* transgene expression is consistent with that of zygotic *Oct4* in early stage embryos. Furthermore, the IF results confirm that the pattern of *EGFP* expression driven by the rabbit *Oct4* promoter resembles that of endogenous *Oct4* gene expression (Fig. 6). Following *Oct4-EGFP* transgene expression, GFP is distributed throughout the entire cell. However, Oct4 is a nucleoprotein; therefore, the GFP signal in Oct4-EGFP transgenic cells covers a much

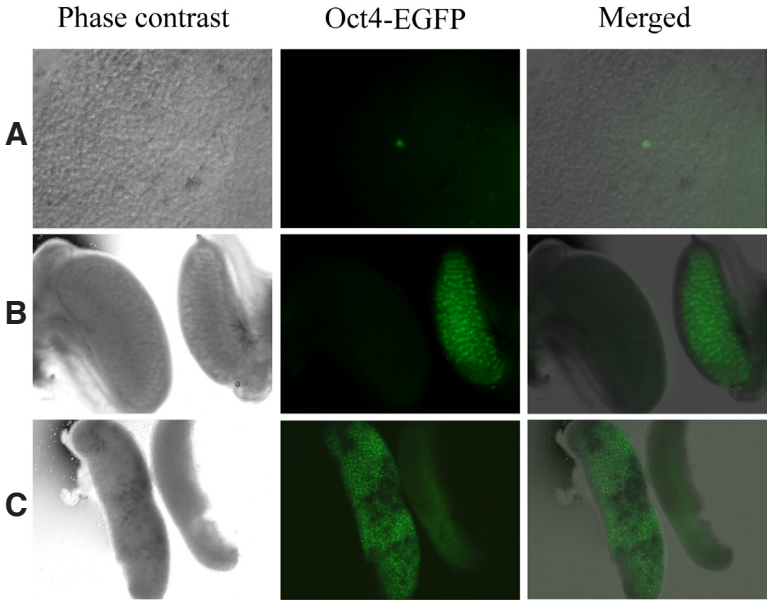

Fig. 7. EGFP expression in fetal tissues or organs at different developmental stages. (A) *EGFP expression in a single cell located between two genital ridges in a 15-dpc fetus.* **(B)** *EGFP expression in the testis of a 27-dpc fetus. Right, testis from a prOG transgenic fetus; left, testis from a normal fetus.* **(C)** *EGFP expression in the ovary of a 27-dpc fetus. Right, ovary from a normal fetus; left, ovary from a prOG transgenic fetus.*

larger area than does nuclear Oct4 immunofluorescence staining. Nevertheless, the expression patterns of the Oct4 reporter and the endogenous *Oct4* gene are similar.

Germline EGFP expression analysis was done using isolated 15-dpc and 27-dpc fetuses. Surprisingly, no green fluorescence was detected in the genital ridges, although it was observed in several cells located between the two genital ridges in the fetus at 15 dpc (Fig. 7A). These green fluorescence cells may be PGCs that have not entered the genital ridges, but we cannot explain the absence of green fluorescence in the genital ridges. However, green fluorescence was clearly detected in testes and ovaries isolated from a 27-dpc fetus (Fig. 7B,C). The EGFP expression pattern is consisted with recent Q-PCR data of rabbit *Oct4* (Daniel-Carlier *et al.*, 2013). The level of Oct4 expression was low in the early stage (at 14 dpc) differentiated gonad and the maximum level of expression was found at around 24–28 dpc in both testes and ovaries. In contrast, OCT4 expression is downregulated in female mice at 14.5 dpc (Pesce *et al.*, 1998) and GFP expression driven by the murine *Oct4* promoter is undetectable at 17.5 dpc (Yoshimizu *et al.*, 1999). These results show that the timing of OCT4 protein expression differs between mouse and rabbit fetal development.

We have therefore demonstrated that the *Oct4-EGFP* transgene is an appropriate method for studying *Oct4* expression in pluripotent cells. In conclusion, we have successfully produced an *Oct4-EGFP* transgenic rabbit, which provides a useful tool to monitor pluripotency in rabbit pluripotent cells.

Materials and Methods

Reagents

All media were purchased from Gibco (Grand Island, N.Y. USA) and all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated. All animal experiments including animal care, the SCNT protocol, embryo transfer, and cesarean sections were carried out according to guidelines of the Ethics Committee of Shanghai Jiao Tong University, School of Medicine.

Reporter plasmid construction

The rabbit *Oct4* promoter (approximately 3.2 kb; GenBank NW_003159279.1) and mouse *Oct4* promoter (approximately 2.3 kb; GenBank AJ297528.1) were PCR amplified using the following primers:

rOCT4-F, 5´-GCGattaatAGACCCAGGAGACTCAAAG-3´; rOCT4-R, 5´-ATAgctagcGGGGGAAGGAGGGCGCCCCGC-3´; mOCT4-F, 5´-AGCTattaatACGCTTAGGGCTAACCTGG-3´; and mOCT4-R, 5´-CTAgctagcTGGTGGAAAGACGGCTCAC-3´.

Genomic DNA was isolated from New Zealand rabbit and C57BL/6J mouse fetal fibroblast cells using a MiniBEST Universal Genomic DNA Extraction Kit (TaKaRa, Dalian, China). Premix long and accurate (LA) PCR (TaKaRa) was performed with the following cycling conditions: 10 min at 98°C; 35 cycles of 98°C for 10 s, 68°C for 30 s, and 72°C for 3 min; and 72°C for 10 min. The amplified *Oct4* promoter was digested by *Ase*I and *Nhe*I, the *CMV* promoter in the pEGFPC1 vector (catalog no. 6084-1, Clontech) was removed by *Ase*I and *Nhe*I digestion, and the *Oct4* promoter was then cloned into the pEGFPC1 vector. The prOG and pmOG vector sequences were confirmed by restriction digest and DNA sequencing.

Transfection of mouse E14 cells

Mouse E14 stem cells (obtained from Dr. Junling Liu) were cultured in DMEM/F12 supplemented with 100× NEAA, 2mM glutamine, 0.1mM β-mercaptoethanol, 15% fetal bovine serum (FBS), and 100 U/mL leukemia inhibitory factor without feeder cells. For transfection, approximately 1×10^5 cells E14 cells were seeded into 3.5-cm dishes. One day later, cells were transfected with 2 μg plasmid DNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's specifications. After 48 h, cells were observed under fluorescence microscopy (IX71 equipped with UV: BH2-RFL-T3 major in 488 μm, Olympus, Japan). Stably transfected E14 clones were obtained by selection with 200 μg/mL G418 for 5-8 days, isolated mechanically, and maintained in culture.

Transfection of rabbit ESCs

Rabbit ESCs were isolated and cultured as previously reported with some modifications (Fang *et al.,* 2006). The culture medium ESM consisted of 78% DMEM/F-12, 20% Knockout serum replacement (Invitrogen, Carlsbad, CA), 2 mM L-glutamine, 1% nonessential amino acids and 0.1 mM β-mercaptoethanol was conditioned with mouse embryonic fibroblasts as reported (Denning *et al.*, 2006, Hannoun *et al.*, 2010). 4–8 ng/ml human recombinant bFGF (basic fibroblast growth factor) was added to the ESM before use. For transfection, confluent rabbit ESCs were seeded at a 1:5 dilution into 3.5-cm dishes coated by Matrigel (Becton Dickinson Biosciences Clontech, Palo Alto, CA, USA). One day later, cells were transfected with 2 μg plasmid DNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's specifications. After 48 h, cells were observed under fluorescence microscopy and 200μg/mL G418 was added to the culture medium for selection.

Establishment of transfected fibroblasts

The isolation, primary culture, and transfection of fibroblasts from a male New Zealand rabbit were described previously (Li *et al.*, 2006, Li *et al.*, 2009). Fibroblasts were cultured in DMEM/F12 supplemented with 10% FBS. For transfection, approximately 1×10^5 fibroblasts/well were seeded into 24-well tissue culture plates. The prOG plasmid was transfected into fibroblasts using Lipofectamine 2000. After 24 h, cells were trypsinized and transferred into four 48-well plates. When cells reached about 40% confluence, 1 mg/mL G418 was added to the culture medium. After 7–10 days of selection, surviving clones were analyzed by PCR and used for SCNT.

Somatic cell nuclear transfer and embryo transfer

The SCNT procedure was carried out as described previously (Li *et al.*, 2006), with some modifications. Briefly, oocytes were collected from superovulated mature white New Zealand female rabbits. Denuded oocytes were enucleated by removing the first polar body and the associated metaphase plate under an inverted microscope equipped with a Spindle View system (Cambridge Research & Instrumentation Inc., USA). prOG transgenic fibroblasts cultured in serum starvation conditions (DMEM supplemented with 0.5% FBS) for 2–4 days were used as donor cells. Donor cells were inserted into the perivitelline space of enucleated oocytes. Reconstructed embryos were electrofused in fusion buffer using two pulses (25 μs DC 2.0 KV/cm) and cultured in mRD (RD medium supplemented with 10% FBS, 2 mM glutamine, 223 μM sodium pyruvate and 100 μM NEAA). SCNT embryos were activated after 30 min by a second identical set of electric pulses and then cultured in mRD supplemented with 5 μg/mL cycloheximide and 2 mM 6-dimethylaminopurine for 1h. Finally, embryos were incubated with mRD for 1 h, followed by incubation in B2 medium prepared as previously reported (Staessen *et al.*, 1998) and supplemented with 5% FBS. Reconstructed embryos were either cultured *in vitro* to the blastocyst stage or transferred to pseudopregnant recipients. *EGFP* expression in embryos was detected by fluorescence microscopy. Between 8 and 15 reconstructed embryos at the 4 to 8-cell stage were transferred into each oviduct of recipients through the infundibulum. Pregnancies were assessed after 14 and 29 days by palpation. Cesarean section was performed 31 days after embryo transfer.

PCR analyses

Genomic DNA was isolated from stably transfected cell lines and transgenic rabbits with a MiniBEST Universal Genomic DNA Extraction Kit (TaKaRa). PCR amplification was performed with Premix LA Taq Version 2.0 (TaKaRa) and the primers Oct4JDF (5'-AGACCCAGGAGACTCAAAG-3')

and Oct4JDR (5'-CGATTTCGGCCTATTGGTT-3'). PCR conditions were: 10 min at 98°C; 35 cycles of 98°C for 10 s, 56°C for 30 s, and 72°C for 5 min; and 72°C for 10 min. DNA fragment size was approximately 4.3 kb. DNA isolated from wild-type rabbits was used as a negative control.

Southern blotting

A phenol-chloroform-isopentanol method was used to extract DNA from three transgenic rabbit fibroblast cell lines and normal rabbit fibroblasts. An *EGFP* DNA probe was amplified from the prOG plasmid with the following primers: GFPprobF, 5´-ACAAGTTCAGCGTGTCCG-3´; GFPprobR, 5´-TTGATGCCGTTCTTCTGC-3´. Amplified DNA fragments (300 ng samples) were DIG labeled using a random primed labeling technique (DIG High Prime DNA Labeling and Detection Starter Kit I; Roche, Mannheim, Germany). Genomic DNA (20 μg) was digested using *Xba*I (TaKaRa). For electrophoresis, a 1% agarose gel was loaded with genomic DNA samples and subjected to 80 V for 4 h. Membrane transfer was carried out using an iBlot dry blotting system (Invitrogen, Israel). After prehybridization, the probe was added and hybridized at 56°C. After 24 h, membranes were washed and blocked, and the hybridized probe was immunodetected with anti-digoxigenin-AP and NBT/BCIP for visualization.

Germline transmission analyses

The male prOG transgenic rabbit was mated with wild-type female rabbits. Fertilized oocytes (F1) were obtained from four the female rabbits at 1 dpc. The embryos were cultured and used for *EGFP* expression analysis. For gonad expression analysis, fetuses were isolated at 15 dpc and 27dpc. The posterior section of the 15-dpc fetus was mounted onto a slide for EGFP expression analysis. Whole testes or ovaries were isolated from 27-dpc fetuses and mounted onto slides for EGFP expression analysis.

Immunofluorescence and confocal imaging

Cultured transgenic E14 and F1 embryos were used for immunofluorescence analysis of endogenous Oct4 expression, as previously reported (Chen *et al*., 2012, Oeda *et al*., 2013) with some modification. First, E14 cells and embryos were fixed by incubation with fresh 4% (v/v) paraformaldehyde in phosphate-buffered saline (PBS) for 1 h at room temperature (RT) and permeabilized by incubating with 0.25% Triton X-100 overnight at 4°C. Next, E14 cells and embryos were blocked in PBS containing 0.3% bovine serum albumin (BSA) for 1 h at RT and incubated with anti-Oct4 antibody (1:150 dilution; MAB4401, Millipore, Billerica, MA, USA) overnight at 4°C. E14 cells and embryos were then washed in three times for 30 min in PBS containing 0.3% BSA and incubated with secondary antibody for 2 h at RT. After another three washes, embryos were incubated with 10 μg/mL Hoechst33342 for 20 min at RT, washed three times, and mounted on slides. E14 cells were observed by fluorescence microscopy and embryos were observed by laser scanning confocal microscopy (Zeiss LSM-510 with a 3-line laser: 488 nm, 543 nm, and 790 nm).

Statement

Semen samples and somatic cells from the Oct4-EGFP transgenic rabbit are available to academic researchers upon request.

Acknowledgements

The study was supported by grants from the Natural Science Foundation of China (Nos. 81170756, 31101048), the Shanghai Natural Science Funding (Nos. 11140901600, 11ZR1418800, 10140901700) and the National Transgenic Foundation of China (2011ZX08008-003).

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5 yr ISI Impact Factor (2011) = 2.959