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Quantitative Monitoring of Pluripotency Gene Activation after Somatic Cloning in Cattle[1]

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ABSTRACT

The development of somatic cell nuclear transfer (SCNT) embryos critically depends on appropriate reprogramming and expression of pluripotency genes, such as *Pou5f1*/*POU5F1* (previously known as *Oct4*/*OCT4*). To study *POU5F1* transcription activation in living bovine SCNT embryos without interference by maternal *POU5F1* mRNA, we generated chromosomally normal fetal fibroblast donor cells stably carrying a mouse *Pou5f1* promoter-driven enhanced green fluorescent protein (EGFP) reporter gene at a single integration site without detectable EGFP expression. Morphologic and quantitative analyses of whole-mount SCNT embryos by confocal microscopy revealed robust initial activation of the *Pou5f1* reporter gene during the fourth cell cycle. In Day 6 SCNT embryos EGFP expression levels were markedly higher than in Day 4 embryos but varied substantially between individual embryos, even at comparable cell numbers. Embryos with low EGFP levels had far more morphologically abnormal cell nuclei than those with high EGFP levels. Our data strongly suggest that bovine SCNT embryos consistently start activation of the *POU5F1* promoter during the fourth cell cycle, whereas later in development the expression level substantially differs between individual embryos, which may be associated with developmental potential. In fibroblasts from phenotypically normal SCNT fetuses recovered on Day 34, the *Pou5f1* reporter promoter was silent but was activated by a second round of SCNT. The restoration of pluripotency can be directly observed in living cells or SCNT embryos from such *Pou5f1*-EGFP transgenic fetuses, providing an attractive model for systematic investigation of epigenetic reprogramming in large mammals.

early development, embryo

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INTRODUCTION

The generation of viable offspring by somatic cell nuclear transfer (SCNT) demonstrated that nuclei of differentiated mammalian somatic cells can be reprogrammed to totipotency by transfer into an enucleated oocyte [1]. The efficiency of animal cloning by SCNT varies considerably among the species studied (reviewed in Shi et al. [2]). The highest success rates of SCNT have been reported in cattle, and the total number of cloned cattle worldwide has recently been estimated in the range of several thousand animals (reviewed in Kues and Niemann [3]). However, even in cattle only a small minority of SCNT embryos develop into an apparently healthy animal, and a large variety of pathologic abnormalities have been reported in bovine SCNT fetuses and live- or dead-born offspring [4–10]. These abnormalities are assumed to originate from incomplete or aberrant epigenetic reprogramming of the somatic donor cell nucleus (reviewed in Shi et al. [2]). Recent studies described abnormal DNA methylation and histone modification patterns in cloned embryos and fetuses that were associated with abnormal embryonic or fetal development [11–13].

Currently, expression of the transcription factor POU5F1 (POU domain, class 5, transcription factor 1; previously known as OCT4) is regarded as the most valid marker for epigenetic reprogramming and pluripotency (reviewed in Pesce and Schöler [14]). There is ample evidence from studies in mice that POU5F1 protein is crucial for normal early embryonic development [15] and for the maintenance of pluripotency in embryonic stem cells [16]. Blastocysts from *Pou5f1* knockout mice do not develop a developmentally competent inner cell mass (ICM; [15]). The cellular amount of POU5F1 protein was shown to crucially affect the differentiation fate of mouse embryonic stem cells [16], providing further substantial evidence that POU5F1 is a key regulator of early embryonic cell differentiation. Moreover, studies in mice have shown that successful reprogramming of a somatic donor cell nucleus after SCNT critically depends on the activation and adequate expression of the *Pou5f1* gene: both analysis of endogenous *Pou5f1* mRNA expression [17] and observation of the *Pou5f1* promoter activity using an enhanced green fluorescent protein (EGFP) reporter gene construct [18] led to the suggestion that aberrant expression of the *Pou5f1* gene might be a major cause for impaired development of cloned mouse embryos [18]. The *Pou5f1*-EGFP reporter construct GOF18-ΔPE-EGFP containing 9 kb of promoter sequences of the mouse *Pou5f1* gene was shown to reflect the expression of the endogenous *Pou5f1* gene in transgenic mouse embryos [19]. Since the genomic organization and regulatory sequences are

highly conserved between the mouse *Pou5f1* gene and its bovine and human orthologues [20, 21], it was assumed that the mouse *Pou5f1* promoter-driven EGFP reporter construct also should be regulated properly in cattle and other mammals. After microinjection of the GOF18-ΔPE-EGFP reporter construct into bovine and porcine zygotes, the resulting blastocysts from both species showed EGFP expression, demonstrating functional conservation of the *Pou5f1* promoter region between mice and other mammalian species [22]. However, the reporter gene expression patterns observed after microinjection into zygotes were strongly influenced by inconsistent and unstable integration and/or mosaicism [22], and thus were difficult to interpret.

To create a strategic tool for reprogramming research in large mammals we decided 1) to transfect bovine fetal fibroblasts (BFFs) with the *Pou5f1* reporter construct GOF18-ΔPE-EGFP; 2) to use cell clones stably carrying the construct at a single chromosome locus for SCNT; 3) to analyze the expression of the *Pou5f1*-EGFP reporter gene construct and the endogenous *POU5F1* mRNA in SCNT embryos at the temporal, spatial, and quantitative level; and 4) to investigate the in vitro and in vivo developmental potency of *Pou5f1*-EGFP transgenic SCNT embryos.

MATERIALS AND METHODS

Cell Culture and Transfection

All animal experiments were carried out according to the institutional rules and with permission of the responsible veterinary authority.

Cattle were bred and raised at the Institute of Molecular Animal Breeding and Biotechnology (Ludwig-Maximilians University). Fibroblasts from a Day 42 bovine fetus (BFF116) were cultured in Dulbecco modified Eagle medium (DMEM; Invitrogen, Karlsruhe, Germany) supplemented with 10% (v/v) fetal calf serum (PAA, Pasching, Austria), 293 mg/l L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (PAA), 0.1 mM 2-mercaptoethanol, and 1% (v/v) nonessential amino acids (PAA). Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 in air.

The reporter gene construct GOF18-ΔPE-EGFP [22, 23] includes the EGFP gene flanked by a 9-kb fragment of the murine *Pou5f1* upstream region with a deletion in the proximal enhancer (PE), and a 9-kb fragment containing the nontranscribed murine structural *Pou5f1* gene. The 21.2-kb fragment GOF18-ΔPE-EGFP was released from the vector backbone by *Not*I digestion and was purified with QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) after gel electrophoresis.

BFF116 cells (5×10^5) were cotransfected with GOF18-ΔPE-EGFP and the neomycin resistance cassette FRT-neo-FRT (kindly provided by Jessica Gorski and Kevin R. Jones, University of Colorado at Boulder, Boulder, CO) by electroporation in Human Dermal Fibroblast Nucleofector Solution (Amaxa, Cologne, Germany) using the Nucleofector device (Amaxa) and program U23. Three days after electroporation the cells were trypsinized and seeded in selection medium (1.0 mg/ml G418) at 2000 cells/well in 96-well plates. Selection medium was changed every other day. After 1 wk, resistant cell colonies were transferred to replica plates for genotyping, karyotype and fluorescent in situ hybridization (FISH) analysis, and cryopreservation. Bovine fetal fibroblasts that were lentivirus transduced with a reporter gene driven by the PGK1 housekeeping gene promoter [24] were used as a positive control for EGFP detection.

PCR Analysis of GOF18-ΔPE-EGFP Transgenic Cell Clones (BFFGOF)

Genomic DNA of G418-resistant cell clones was extracted by digestion with proteinase K (Roche, Mannheim, Germany). Primers used to detect the presence of the reporter gene GOF18-ΔPE-EGFP were GOFGFP3 (forward) 5′-ccctaggtgagccgtctttcc-3′ and GOFGFP4 (reverse) 5′-ccttgatgccgttcttctgct-3′. Twenty-microliter reactions contained 2 μl of 10× PCR buffer (Qiagen), 4 μl Q-solution (Qiagen), 1.25 μl $MgCl_2$ (25 mM), 1 μl dinucleotide triphosphates (1 mM), 1 μl primer (2 μM), and 0.1 μl Taq polymerase (5 U/μl; Qiagen). The cycling conditions for PCR reactions were: 3 min at 94°C, 35 cycles with 30 sec at 94°C, 30 sec at 63°C, 1 min at 72°C, and 10 min at 72°C for final extension.

Chromosome Analysis and FISH

Transgenic cell clones at passages 10–13 were harvested for karyotyping and transgene integration analysis by FISH. Metaphase spreads were prepared following standard protocols [25].

To generate a FISH probe specific for the reporter gene construct GOF18-ΔPE-EGFP, 10 DNA fragments ranging in size from 500 to 724 bp and spanning a total of ~5.7 kb (Fig. 1A) were amplified by PCR from plasmid DNA. Primers were designed using Primer Express software (Applied Biosystems, Darmstadt, Germany). The transgene-specific DNA fragments were labeled with TAMRA-dUTP (Applied Biosystems) in 20-μl reactions by either single or multiplex PCR from plasmid DNA or aliquots of unlabeled PCR products obtained by an intermediate amplification step. As a positive control, we included a DNA probe labeled with biotin-dUTP (Roche) identifying locus D6Z1 on bovine chromosome 6, which has been described in detail elsewhere [26]. Both the TAMRA-labeled, transgene-specific fragments and the biotin-labeled control probe were mixed, ethanol precipitated, and resuspended in hybridization buffer (Dako, Glostrup, Denmark).

The probe DNA was denatured at 75°C for 10 min and cooled on ice until application. Chromosome preparations were denatured in 70% formamide/2× SSC, pH 7.0, at 70°C, dehydrated in a series of increasingly concentrated ethanol, and air dried before probe loading. Hybridization for 72–96 h at 37°C was followed by high-stringency washes for 2 × 5 min at 60°C in 0.1× SSC. Hybridization of the biotin-labeled positive control was detected by Avidin Alexa Fluor 488 (Invitrogen). Chromosomes were counterstained with 4′,6′-diamidino-2-phenylindole (DAPI) and mounted with Vectashield antifade solution (Vector Laboratories, Burlingame, CA). Metaphase images were captured using a confocal laser scanning microscope (LSM 510 Meta; Zeiss, Göttingen, Germany) and a 63× PlanApochromat (numerical aperture 1.4) oil immersion objective.

Somatic Cell Nuclear Transfer

The originally transfected BFF clones were used for first-round SCNT at passages 9–11, and fibroblasts derived from transgenic fetuses were used at passages 4–6 for second-round SCNT. The nuclear transfer procedure has been described in detail previously [27]. Cloned embryos were cultured in 100-μl drops of synthetic oviduct fluid supplemented with 5% (v/v) estrous cow serum at 39°C in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2, covered by paraffin oil.

Whole-Mount Preparation of SCNT Embryos and Confocal Laser Scanning Microscopy

EGFP expression of BFFGOF12 SCNT embryos was analyzed on Days 4 and 6. EGFP fluorescence was initially checked using an inverted epifluorescence microscope (Axiovert 200M; Zeiss) equipped with an appropriate narrow bandpass filter set for excitation at 470/20 nm and emission at 505–530 nm (Zeiss). Digital images were taken with a 20× objective and a color CCD camera (AxioCam HR; Zeiss). Subsequently, the embryos were fixed with 4% (w/v) paraformaldehyde in PBS for 30 min, followed by washing with PBS containing 1 mg/ml polyvinyl pyrrolidone. After staining the cell nuclei with 1 μg/μl Hoechst 33342 for 30 min, embryos were mounted in Vectashield antifade solution on coverslips in such a way that the three-dimensional structure of the specimen was maintained. Stacks of optical serial sections (optical thickness 1 μm) were recorded using a confocal laser scanning microscope (LSM 510 Meta; Zeiss) with a 40× PlanApochromat (numerical aperture 1.3) oil immersion objective. For excitation of Hoechst 33342 and EGFP, laser lines of 364 nm and 488 nm were used, respectively. The resulting fluorescence emissions were detected through emission bandpass filters at 385–470 nm (Hoechst 33342) and 505–530 nm (EGFP). In addition, the emission spectrum of each embryo obtained by excitation at 488 nm was analyzed and compared to the emission spectrum of EGFP using a polychromatic multichannel detector (Meta detector; Zeiss) to clearly differentiate EGFP fluorescence from autofluorescence.

The EGFP fluorescence intensity of individual embryos was assessed on central optical sections, including all pixel intensity values of the total area occupied by blastomeres. This was done using ImageJ software (National Institutes of Health, Bethesda, MD). Median intensity values were used to compare the EGFP fluorescence of individual embryos and to correlate it with the number of cell nuclei. In each embryo we determined 1) the total number of cell nuclei and 2) the number of decaying (i.e., fragmented, pyknotic, and apoptotic) nuclei by comparing each optical serial section from bottom to top to the subjacent section.

Whole-Mount RNA In Situ Hybridization

Localization of *POU5F1* transcripts in SCNT bovine embryos was performed as previously described [28]. For in vitro transcription of a digoxigenin-labeled bovine *POU5F1* mRNA-specific antisense riboprobe, a 517-nucleotide fragment (nucleotides 808–854, 1184–1342, and 1448–1755; GenBank accession no. AF022987) was amplified from bovine blastocysts by

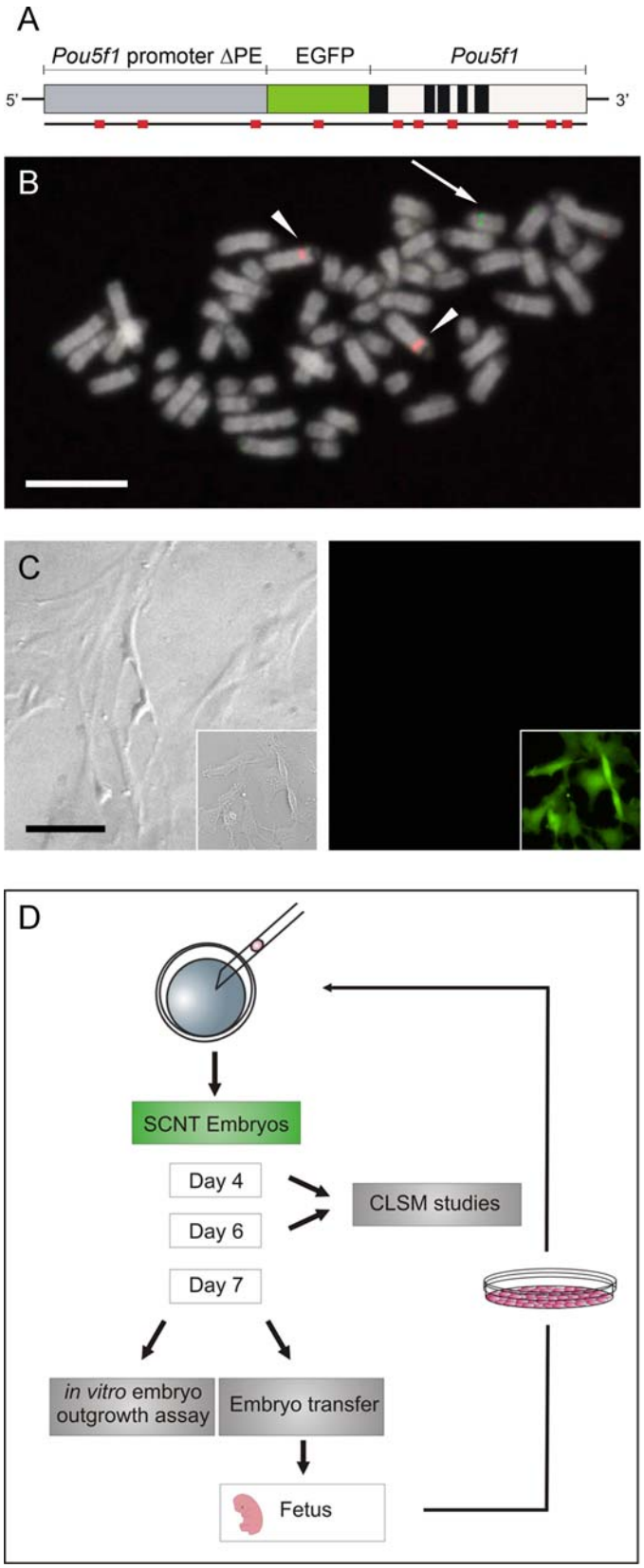

FIG. 1. **A**) The reporter gene construct GOF18-ΔPE-EGFP (21.2 kb), which contains EGFP flanked by a 9-kb fragment of the murine *Pou5f1* upstream region with a deletion in the proximal enhancer (PE) and a 9-kb fragment containing the (nontranscribed) structural *Pou5f1* gene. Exons are represented by black boxes. The red boxes mark the target sites for the transgene-specific FISH probe. **B**) FISH on metaphase spreads demonstrating a single-locus integration of the reporter gene construct in cell clone BFFGOF12 of transfected BFFs. The hybridization signal on the GOF18-ΔPE-EGFP transgene (green) is indicated by an arrow. A FISH probe identifying locus *D6Z1* on bovine chromosome 6 (red, marked by arrowheads) served as a control for hybridization efficiency. Bar = 10 μm. **C**) In the transfected BFFGOF12 cells no EGFP fluorescence was detectable (left, bright field; right, fluorescence image). The insets show BFFs carrying a reporter gene driven by the PGK1 housekeeping gene promoter; these were used as a positive control for EGFP detection. Bar = 100 μm. **D**) Experimental design: fetal fibroblasts with a single-locus integration of GOF18-ΔPE-EGFP but without detectable EGFP expression were selected for first-round SCNT. Mouse *Pou5f1* promoter-driven expression of EGFP was analyzed in SCNT embryos on Days 4 and 6 by confocal laser scanning microscopy (CLSM). Day 7 SCNT embryos were used for in vitro outgrowth formation assay and embryo transfer. GOF18-ΔPE-EGFP transgenic fibroblasts isolated from SCNT fetuses showed silencing of the reporter gene construct and were used for second-round SCNT.

RT-PCR and was subcloned into pCRII TOPO (Invitrogen, Carlsbad, CA). For whole-mount in situ hybridization, embryos (Days 4, 6, and 7) were fixed in 4% paraformaldehyde/0.1% glutaraldehyde at room temperature for 30 min. Fixed embryos were treated with 10 μg/ml proteinase K in PBS supplemented with 10% Tween-20 (PBT) for 15 min at room temperature, and then with 2 mg/ml glycine in PBT for 10 min at room temperature. Prehybridization was performed in hybridization solution (50% formamide; 5× SSC, pH 4.5; 0.1% Tween-20; 50 μg/ml heparin) containing 100 μg/ml tRNA and 100 μg/ml salmon sperm DNA for 2 h at 55°C, and hybridization with riboprobe in hybridization solution containing tRNA and salmon sperm DNA was performed overnight at 58°C. Stringent posthybridization washes were performed three times for 20 min each in 2× SSC, pH 4.5; 50% formamide; and 0.1% Tween-20 at 59°C. After hybridization, embryos were incubated in blocking solution (10 mM maleic acid, 15 mM NaCl, 1% blocking reagent [Roche 11 096 176 001], and 2% sheep serum), and then were treated with 1:2000 anti-digoxigenin-AP Fab fragments (Roche 11 093 274 910) in blocking solution overnight at 4°C. After antibody treatment, embryos were washed in a solution containing 10 mM maleic acid, 15 mM NaCl, 10% Tween-20, and 2 mM levamisole to block endogenous alkaline phosphatase. For color reaction, embryos were incubated in 1:50 NBT/BCIP Stock Solution (Roche 1 681 451) in solution containing 100 mM NaCl, 100 mM Tris, 50 mM $MgCl_2$, 0.1% Tween-20, and 2 mM levamisole for 60–70 min at room temperature. Embryos were washed and mounted in PBS containing 25 mM EDTA and were observed under an inverted microscope.

Outgrowth Formation Assay

Mitomycin C-treated STO cells (feeder cells) were plated on gelatinized four-well plates (2×10^4 cells/well) in fibroblast cell culture medium (see above). Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 in air. Day 7 SCNT embryos from cell clone BFFGOF12 were placed on the feeder cells and incubated at 39°C in a humidified atmosphere of 5% CO_2 in air until Day 15. The culture medium was changed both before plating the embryos on the feeder cells and on Day 13. Outgrowth formation was observed every other day.

Embryo Transfer and Derivation of Transgenic Fetal Fibroblasts

EGFP-positive Day 7 blastocysts were transferred to synchronized recipients as described previously [24]. Pregnancy was diagnosed by ultrasonography on Day 28. Pregnancies were terminated on Day 34 after SCNT. The recovered fetuses were carefully examined for malformations and subsequently processed to establish transgenic fetal fibroblast cultures [27]. In brief, after removal of head, tail, and visceral organs, the remaining fetal tissue was finely minced and dissociated with PBS containing 1.0 g/l trypsin and 0.4 g/l EDTA (PAA) for 7 min at 37°C. The resulting cell suspension was washed with cell culture medium (see above) and seeded on cell culture plates. Primary cultures were grown for 7 days and cryopreserved in multiple aliquots.

RESULTS

Stable Transfection of BFFs with the GOF18-ΔPE-EGFP Reporter Gene Construct

PCR screening of 113 BFF colonies resistant to the selection antibiotic revealed a GOF18-ΔPE-EGFP-specific amplicon in 16 clones. As expected for differentiated somatic cells, none of

TABLE 1. EGFP expression driven by the mouse *Pou5f1* promoter in Day 4 bovine SCNT embryos from bovine fetal fibroblasts (cell clone BFFGOF12).

No. of nuclei	No. of embryos	EGFP expression (%)
1–4	38	0
5–8	8	0
9–16	13	9 (69%)
17–30	14	14 (100%)

the fibroblast clones showed detectable (*Pou5f1* promoter-driven) EGFP expression (Fig. 1C). In a pilot experiment, six of these clones were used for nuclear transfer, and the resulting SCNT embryos were checked for EGFP fluorescence at the morula or blastocyst stage. EGFP expression was observed in cloned embryos from three of the six transfected BFF clones, demonstrating that the mouse *Pou5f1* promoter of the GOF18-ΔPE-EGFP reporter gene construct could be activated in early bovine SCNT embryos. The three BFF clones leading to EGFP-expressing embryos were subjected to karyotyping and transgene integration analysis by FISH. Cell clone BFFGOF12, which had a normal karyotype (60, XX) and a single transgene integration site, was chosen for studies on the reporter gene expression in SCNT embryos (Fig. 1B).

Consistent Initiation of GOF18-ΔPE-EGFP Expression in Bovine SCNT Embryos

In total, 200 nuclear transfers using the transfected clone BFFGOF12 were performed in three independent experiments. The fusion rate was 96% ± 1%. In each experiment, the nuclear transfer complexes were randomly divided into two groups for evaluation on Days 4 and 6. In total, 73 embryos from Day 4 and 53 embryos from Day 6 were evaluated by epifluorescence and confocal laser scanning microscopy.

True EGFP fluorescence was validated and clearly separated from autofluorescence by analyzing the emission spectrum of all *Pou5f1*-EGFP transgenic embryos and nontransgenic control embryos at 488-nm excitation using a polychromatic multichannel detector (supplemental Figure S1, available online at www.biolreprod.org). Table 1 summarizes numbers of nuclei and EGFP expression in Day 4 embryos. No EGFP expression was detected in embryos with fewer than nine nuclei, clearly indicating that the *Pou5f1* promoter was not active before passing the fourth cell division. A total of 9 of 13 embryos contained between 9 and 16 nuclei and exhibited EGFP fluorescence in at least one or more blastomeres, some of them with different fluorescence intensities among the blastomeres. As a representative example, an SCNT embryo with 12 blastomeres showing different levels of EGFP expression is shown in Figure 2, A and A′.

Developmental Heterogeneity and Variable GOF18-ΔPE-EGFP Expression in Day 6 SCNT Embryos

The morphology, the number of cell nuclei, and the level of EGFP expression of Day 6 embryos varied in rather broad ranges: 31 of 53 embryos evaluated on Day 6 had fewer than nine nuclei and did not exhibit any EGFP expression. However, EGFP fluorescence was unequivocally detected in all Day 6 embryos with nine or more nuclei and was considerably stronger than in Day 4 embryos (Fig. 2, B and B′). Figure 3 (central diagram) shows the median fluorescence intensity values assessed on central optical sections of embryos plotted against the numbers of nuclei. There was no significant correlation ($r = 0.34$, $P = 0.12$) between the median fluorescence intensity and the number of nuclei. In general, nuclei of embryos exhibiting high EGFP fluorescence (Fig. 3, upper image panel) appeared to be more homogeneous in shape and size compared with those of embryos with low EGFP expression (Fig. 3, lower image panel). Therefore, we compared the incidence of nuclear abnormalities (condensed/fragmented nuclei) between SCNT embryos with high and low EGFP fluorescence at similar cell numbers. Notably, embryos with low EGFP expression exhibited a significantly higher proportion (5.8%–9.4%; mean: 8.0%) of abnormal nuclei

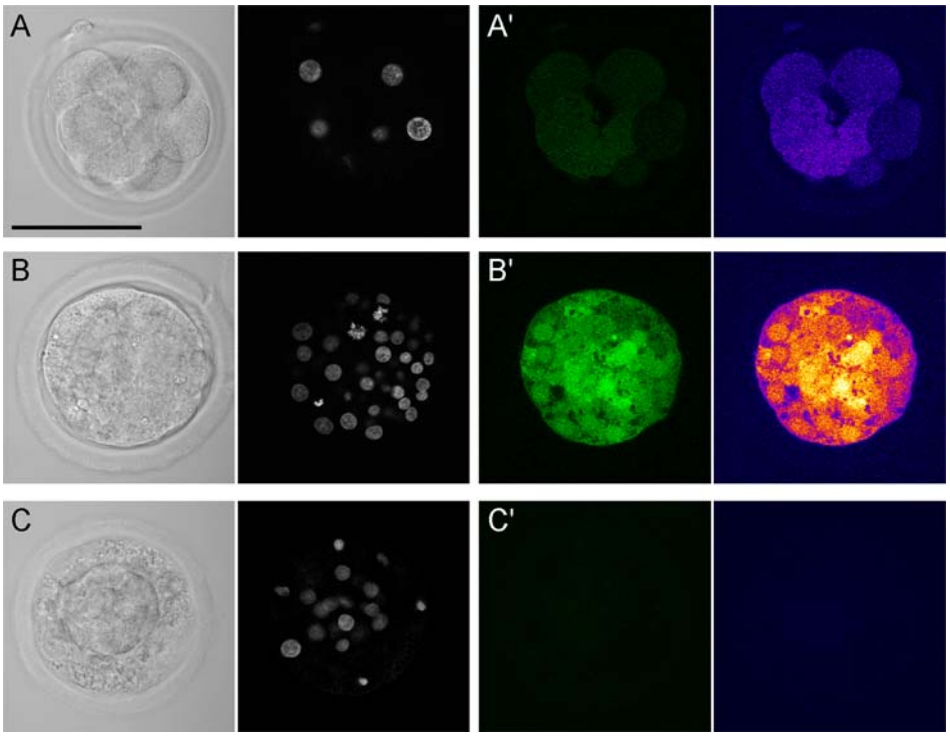

FIG. 2. Analysis of mouse *Pou5f1* promoter-driven EGFP expression in bovine SCNT embryos by CLSM on Days 4 and 6. Each horizontal panel shows single-channel images of an optical section through the center of a representative whole-mount embryo. **A**–**C**) Transmission scan image (left) and the cell nuclei stained with Hoechst 33342 (right). **A′**–**C′**) Linear intensity image (left) and color-coded intensity map (right) of EGFP fluorescence. **A**, **A′**) Day 4 embryo with 12 nuclei: EGFP is detectable in all blastomeres but at different levels in individual blastomeres. A total of 9 of 13 Day 4 embryos with at least nine nuclei showed EGFP fluorescence in at least one or more blastomeres. No EGFP expression was detected in embryos with fewer than eight nuclei, indicating that the *Pou5f1* promoter was not activated before passing the fourth cell division. **B**, **B′**) Highest EGFP fluorescence intensities were measured on Day 6 at the morula/blastocyst stage. **C**, **C′**) Negative control: nontransgenic Day 6 embryo created by in vitro fertilization. Bar = 100 μm.

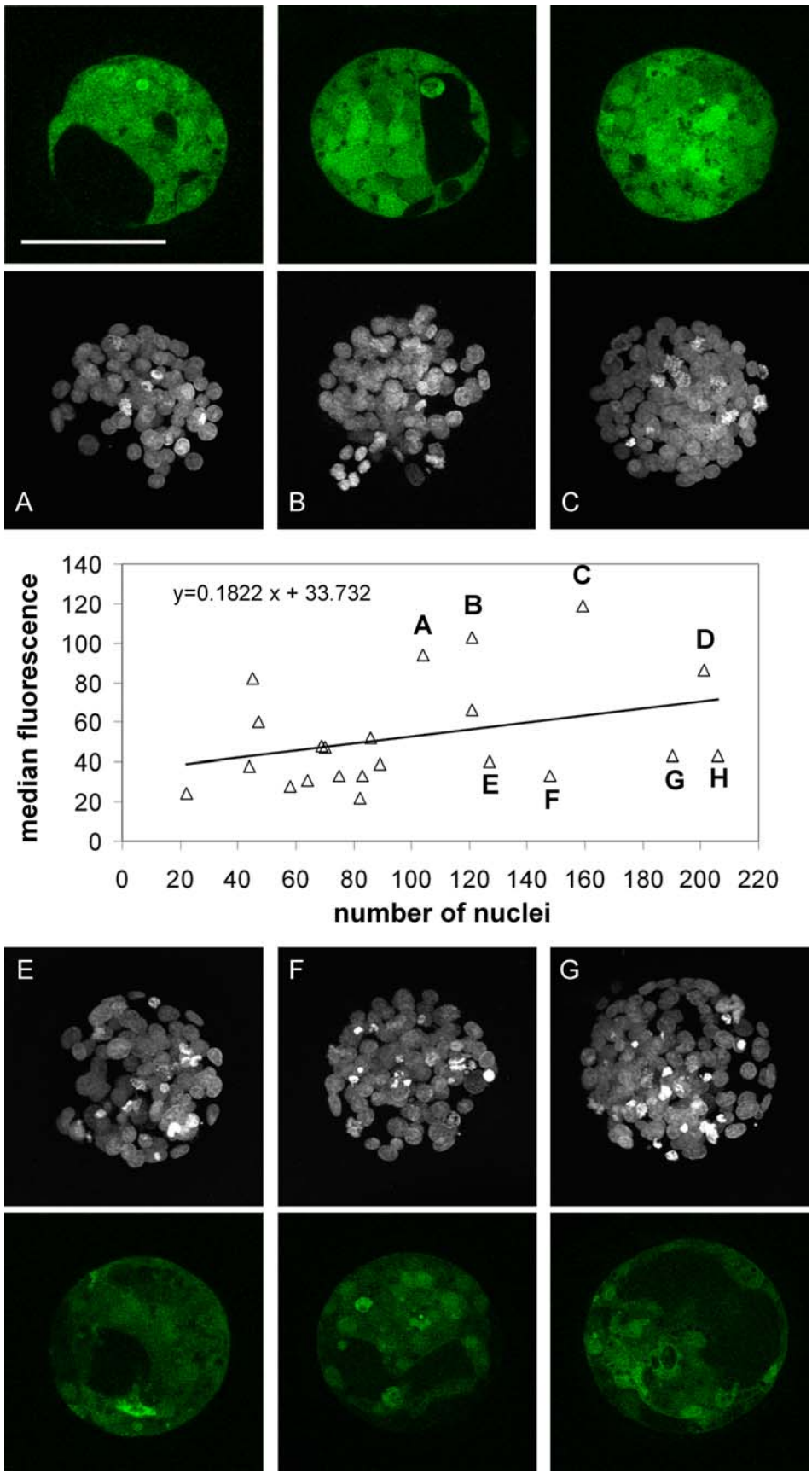

FIG. 3. Analysis of mouse *Pou5f1* promoter-driven EGFP fluorescence intensity in Day 6 SCNT embryos. Central diagram: median intensity values determined on central confocal optical sections are plotted against nuclei numbers. Between the median fluorescence intensities and the embryonic nuclei numbers a weak positive correlation ($r = 0.34$, $P = 0.12$) was found. In embryos with high EGFP expression (values indicated by A–D) the proportion of abnormal nuclei was very small and the cell nuclei appeared more homogeneous in shape and size compared with embryos with a similar number of nuclei but low EGFP expression (values indicated by E–H). The upper and the lower image panels show three embryos with high EGFP fluorescence (denoted A, B, C) and three embryos with low EGFP fluorescence (denoted E, F, and G). The cell nuclei of the embryos was stained with Hoechst 33342 and are displayed as maximum intensity projections of confocal image stacks; EGFP expression is demonstrated on central confocal sections. The letters in the image panels refer to the respective (identically noted) measured values in the central diagram. Bar = 100 μm.

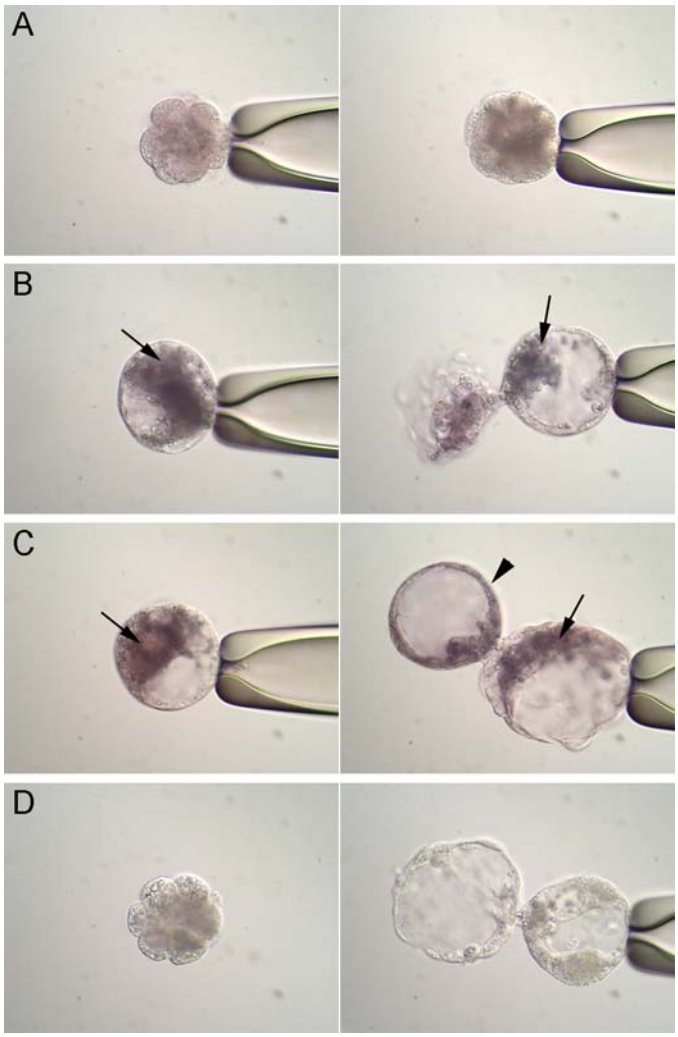

FIG. 4. Analysis of endogenous *POU5F1* transcripts in bovine SCNT embryos derived from BFFGOF12 cells by whole-mount RNA in situ hybridization. **A**–**C**) Hybridization of digoxigenin-labeled antisense RNA probes was detected by NBT/BCIP color reaction. **A**) In Day 4 embryos, low *POU5F1* transcript levels were detected. Embryo on the right at a more advanced stage of development showed a stronger signal than that on the left. **B**, **C**) In Day 6 and Day 7 embryos, higher signal intensities were observed. **B**) Prehatched (left) and hatching (right) Day 6 blastocyst with high *POU5F1* transcript levels in the ICM (arrow). **C**) Prehatched (left) and hatching (right) Day 7 blastocyst with high *POU5F1* transcript levels in the ICM (arrow). Some 50% of Day 6 and Day 7 embryos showed hybridization signals also in the trophectoderm (arrowhead). **D**) Negative controls: Day 4 embryo (left) and Day 6 blastocyst (right) hybridized without riboprobe.

compared with the embryos with high EGFP expression (0.6%–5.0%; mean: 1.8%).

Expression of Endogenous POU5F1 *mRNA in SCNT Embryos*

The presence of endogenous *POU5F1* mRNA was investigated by whole-mount in situ hybridization in 15 SCNT embryos from Day 4, 26 SCNT embryos from Day 6, and 23 SCNT embryos from Day 7 (Fig. 4). *POU5F1* mRNA signal was detected in single Day 4 embryos. Increased signal intensity was observed in all Day 6 and Day 7 embryos; some 50% exhibited *POU5F1* mRNA expression only in the ICM, whereas the remaining embryos showed a positive hybridization signal in both ICM and trophectoderm (TE).

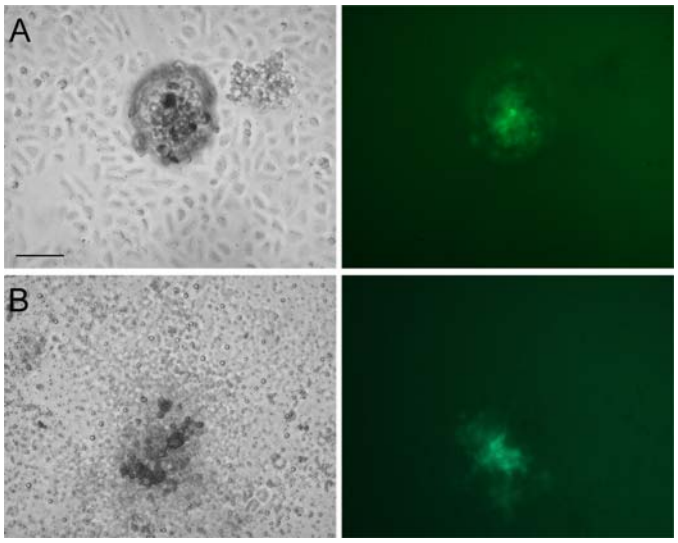

FIG. 5. Outgrowth formation assay with BFFGOF12 SCNT embryos. **A**) Day 13 embryo placed on feeder cells. Left: bright field; right: fluorescence. **B**) Outgrowth formation on Day 15. Left: bright field; right: fluorescence. Bar = 100 μm.

Outgrowth Formation In Vitro

To gain initial information on a potential correlation between level of EGFP expression and the differentiation potential of *Pou5f1*-EGFP transgenic SCNT embryos, we performed in vitro outgrowth experiments on Day 7 blastocysts shortly before or after hatching. In the experiments, nine blastocysts were placed on feeder cells (STO cells) and were cultured until Day 15. Two blastocysts, one hatched and one nonhatched, showing low EGFP fluorescence did not form outgrowths. One hatching and five hatched blastocysts with medium to high EGFP expression attached to the feeder cells and formed outgrowths. In one case, EGFP fluorescence was detected in ICM outgrowth areas until Day 15 after SCNT; in another case, it was detected until Day 13 (Fig. 5). One blastocyst showing strong EGFP fluorescence did not hatch, and thus could not form an outgrowth.

In Vivo Development of SCNT Embryos from BFFs Stably Transfected with GOF18-ΔPE-EGFP and Second-Round SCNT

Transfer of three Day 7 SCNT embryos (early blastocysts) from the transfected clone BFFGOF12 into each of four synchronized recipients resulted in one singleton and one twin pregnancy, which were terminated on Day 34 after SCNT. Three normally developed fetuses without detectable malformations were recovered (Fig. 6A). In fibroblast cultures from the transgenic fetuses, EGFP fluorescence was not detected (Fig. 6, B and C). Thus, the mouse *Pou5f1* promoter was silent in the differentiated somatic fetal cells. As in the originally transfected cell clones, cytogenetic analysis revealed a normal karyotype (60, XX) and a single chromosomal transgene integration site (Fig. 6D). Fibroblasts derived from one transgenic fetus (FeGOF451–1) were used for second-round SCNT to determine whether the *Pou5f1* promoter-driven reporter gene was activated. Indeed, the GOF18-ΔPE-EGFP reporter gene expression was initiated after SCNT (Fig. 6F).

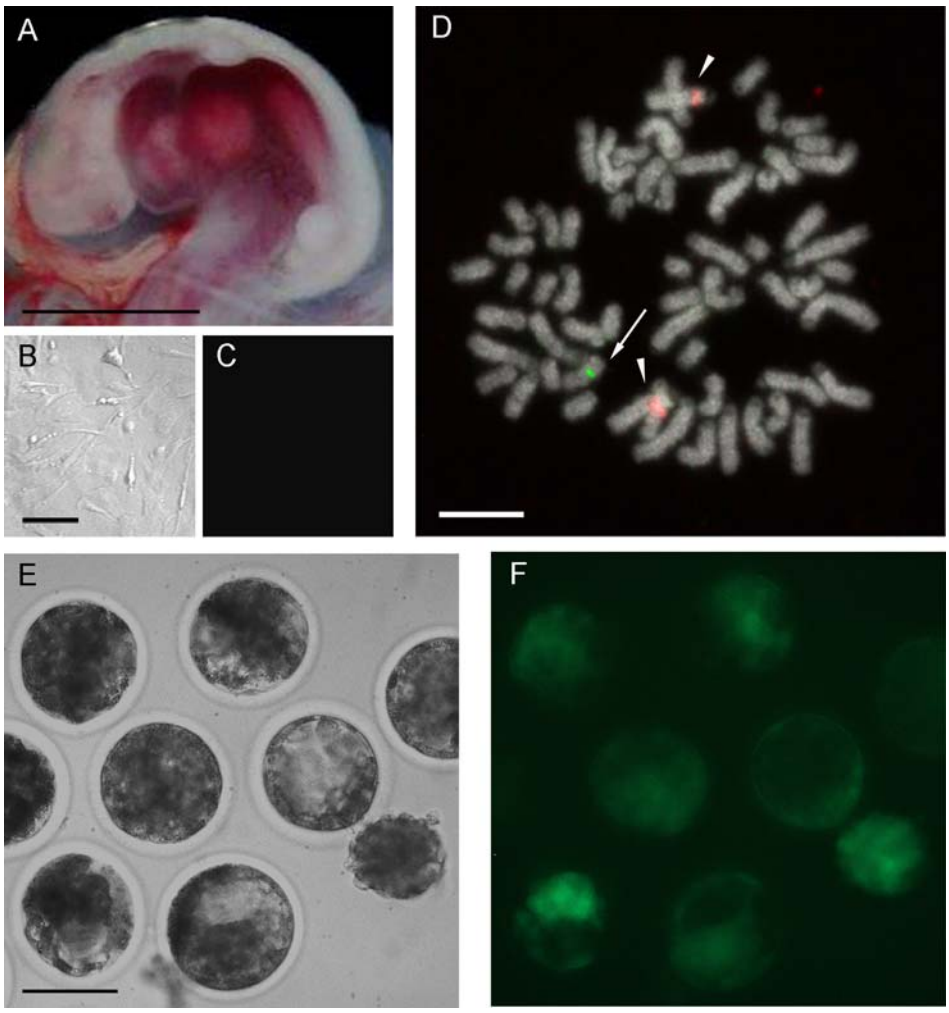

FIG. 6. In vivo development of SCNT embryos from BFFs stably transfected with GOF18-ΔPE-EGFP and EGFP expression in second-round SCNT embryos. **A**) A 34-day-old SCNT fetus from cell clone BFFGOF12. In fibroblasts isolated from this fetus (line FeGOF451–1), no EGFP fluorescence was detectable: bright field (**B**) and fluorescence (**C**) images. The mouse *Pou5f1* promoter had been effectively silenced in the differentiated somatic fetal cells. **D**) FISH on metaphase spreads confirmed the stable single-locus integration of the reporter gene construct in cells isolated from the SCNT fetus. The hybridization signal on the GOF18-ΔPE-EGFP transgene (green) is indicated by an arrow. Red signals (marked by arrowheads) indicate detection of locus D6Z1 on bovine chromosome 6 as a control for hybridization efficiency. Transgenic fibroblasts isolated from the SCNT fetus (line FeGOF451–1) were used for second-round SCNT. EGFP expression driven by the mouse *Pou5f1* promoter was again activated: stereomicroscopic (**E**) and fluorescence (**F**) images from Day 7 embryos. Bar = 6.5 mm (**A**), 10 μm (**B**–**D**), and 100 μm (**E**, **F**).

Notably, when compared to first-round SCNT embryos from the originally transfected cell clone BFFGOF12, second-round SCNT embryos from transgenic FeGOF451–1 fetal fibroblasts developed to blastocysts at a significantly ($P < 0.05$) higher rate (Table 2).

DISCUSSION

The marked differences in the efficiency of SCNT between different mammalian species strongly suggest that in spite of a probably conserved general principle, important aspects of nuclear reprogramming are determined by species-specific mechanisms. Potential factors include differences in the timing of embryonic genome activation (reviewed in Memili and First [29]) and in the temporal/spatial pattern of DNA methylation and histone modifications [11, 30]. The present study used activation of the "pluripotency" gene *POU5F1* as an indicator for nuclear reprogramming after SCNT in the bovine, the species in which this technology currently has the highest efficiency. To facilitate this analysis in living embryos, we used nuclear donor cells with a stable single-site integration of a *Pou5f1*-EGFP reporter gene.

EGFP fluorescence was not detected in embryos with fewer than nine nuclei, but it was detected in all embryos with 16 or more cells, indicating that the *Pou5f1*-EGFP reporter gene was consistently activated between the third and fourth cell cycles. In spite of the markedly lower efficiency of SCNT in the mouse, activation of EGFP expression was observed in more than 80% of embryos cloned from cumulus cells carrying the same reporter gene [18]. However, EGFP expression started one cell

TABLE 2. Efficiency of first round SCNT using originally transfected fetal bovine fibroblasts (BFFGOF12) and second round SCNT using fibroblasts isolated from a GOF18-ΔPE-EGFP transgenic fetus (FeGOF451–1).

Donor cells	Experiments	Oocytes (n)	Fusion rate (%)[c]	Cleavage rate (%)[c,d]	Blastocyst rate (%)[c,e]
BFFGOF12[a]	4	161	93.7 ± 2.6	87.8 ± 4.8	24.7 ± 8.5
FeGOF451–1[b]	3	113	95.5 ± 4.0	61.5 ± 10.0	39.8 ± 4.4*

[a] Cells used at passages 9–11.
[b] Cells used at passages 4–5.
[c] Values are mean ± SD.
[d] Rate is based on fused karyoplast-cytoplast complexes.
[e] Rate is based on cleaved embryos.
* Significantly different between the two donor cell types; $P = 0.02$.

cycle earlier, between the four- and eight-cell stages, which is consistent with the earlier maternal zygotic transition in mouse compared to cattle (reviewed in Memili and First [29]).

Interestingly, a marked variation of EGFP fluorescence intensity was observed in bovine embryos cloned from *Pou5f1*-EGFP-transfected fibroblasts. Low fluorescence levels were associated with an increased proportion of abnormal nuclei. These observations indicate: 1) that further modulation of pluripotency gene expression may occur after initial consistent activation coincident with the major wave of maternal zygotic transition, and 2) that low expression of pluripotency gene may be associated with reduced developmental potential. An association between POU5F1 expression and developmental potential is supported by the lack of outgrowth formation from blastocysts with low *Pou5f1*-EGFP expression.

A key finding in cloned mouse embryos was abnormal spatial distribution of *Pou5f1* mRNA in SCNT mouse blastocysts, 55% of which showed *Pou5f1* expression in both the ICM and the TE. In contrast, ectopic localization of *Pou5f1* transcripts in the TE was observed in only 11% of blastocysts derived by in vitro fertilization and in 4% of in vivo-produced embryos [18]. Our in situ hybridization studies of *POU5F1* transcript localization in cloned bovine embryos revealed about 50% of embryos with *POU5F1* mRNA signals in both the ICM and the TE, whereas the other half of the cloned embryos showed endogenous *POU5F1* transcripts only in the ICM, as was previously described for in vitro-produced bovine blastocysts [28]. Thus, half of the bovine SCNT embryos expressed *POU5F1* transcripts in the TE, an expression pattern that has been shown to be associated with the abnormal development of cloned mouse embryos [18]. The consequences of *POU5F1* mRNA expression in the TE of bovine SCNT blastocysts for further development are not clear. One potential mechanism by which POU5F1 might affect TE cell function is regulation of the expression of interferon tau (IFNT), the major embryonic pregnancy recognition signal in ruminants [31].

Studies in human chorion carcinoma cells transfected with *IFNT1* promoter-luciferase reporter constructs revealed that POU5F1 silences *IFNT* promoters by quenching *ETS2* transactivation [32]. Thus, it will be interesting to compare the levels of IFNT secretion by blastocysts with versus those without *POU5F1* expression in the TE, and to systematically analyze maternal responses to these classes of embryos, such as through holistic transcriptome [33, 34] and proteome studies [35] of endometrium samples from pregnant recipients. It will be of particular interest to study in detail the correlation between the transcription level of *POU5F1* and the expression of *CDX2* and its protein. Recent observations have provided convincing evidence for an essential role of CDX2 protein in the segregation of ICM and TE in mouse embryos by repressing *Pou5f1* and *Nanog* in the TE lineage cells [36].

In spite of similar proportions of *POU5F1*/*Pou5f1* transcript localization in the TE of bovine and mouse SCNT blastocysts, the development in vivo was markedly different between the two species. In our study in cattle, transfer of SCNT blastocysts from *Pou5f1*-EGFP-transfected cells to four recipients resulted in two pregnancies, and three (50%) of the six embryos in pregnant recipients developed into apparently normal fetuses. In mice, transfer of two-cell SCNT embryos from *Pou5f1*-EGFP transgenic cumulus cells to recipients resulted in a comparable pregnancy rate (24 [59%] of 41), but only 0.8% (7 of about 850) of two-cell embryos or about 11% of blastocysts (9% blastocyst rate based on two-cell embryos) in pregnant recipients developed into a fetus (Day 10 after conception; [18]). Taken together, these findings indicate that the presence of *POU5F1*/*Pou5f1* mRNA in the TE of cloned blastocysts does not explain the marked difference in the efficiency of SCNT between mouse and cattle.

Careful examination of the three SCNT fetuses derived from *Pou5f1*-EGFP-transfected fibroblasts (BFFGOF12) at low magnification revealed that they were free of malformations and were appropriately developed for the gestational age. EGFP expression was not detectable, demonstrating that the mouse *Pou5f1* promoter was silent in differentiated cells. However, *Pou5f1*-EGFP transgenic fibroblasts isolated from these fetuses could be reprogrammed to express EGFP by a second round of nuclear transfer. This further confirmed that *Pou5f1*-EGFP transgenic fetuses are a valuable source of cells to systematically investigate epigenetic reprogramming of different cell types, either by in vitro systems or by SCNT. Interestingly, the rate of development to blastocyst was higher in second-round SCNT using transgenic fetal fibroblasts than it was in first-round SCNT experiments from *Pou5f1*-EGFP-transfected fibroblasts. This observation indicates that transfection and selection procedures may negatively affect the potential of donor fibroblasts for SCNT, but the underlying changes can be rescued during the development of a cloned fetus, which provided transgenic nuclear donor cells with a higher developmental potential.

An interesting application of *Pou5f1*-EGFP transgenic cloned blastocysts may be the derivation of pluripotent stem cells in the bovine. Many attempts to derive pluripotent embryonic stem (ES) cells in the bovine have resulted thus far only in ES-like cells, which are characterized by the expression of certain marker genes and by in vitro differentiation properties (reviewed in Prelle et al. [37]). Two studies produced chimeric animals, demonstrating the differentiation potential of bovine ES-like cells in vivo [38, 39]; however, contribution to the germline was not shown. Our in vitro outgrowth experiments demonstrated continuous EGFP expression in some of the outgrowths from *Pou5f1*-EGFP SCNT embryos. Future studies need to clarify whether these cells provide a superior source for the derivation of pluripotent cell lines.

In summary, we showed that *Pou5f1*-EGFP transgenic fibroblasts with a single chromosomal integration site are a suitable model to monitor *Pou5f1* promoter activation in bovine SCNT embryos. We demonstrated that virtually all SCNT embryos developing beyond the 16-cell stage activated the *Pou5f1* promoter. However, the level of *Pou5f1*-EGFP expression was highly variable between individual embryos. Moreover, we obtained considerable evidence that the level of *Pou5f1*-EGFP expression is associated with parameters of embryo quality and viability, such as the proportion of abnormal cell nuclei and the ability to form outgrowths in vitro. Chromosomally and phenotypically normal fetuses with a stable single locus insertion of the *Pou5f1*-EGFP reporter gene construct permitting activation and silencing of the *Pou5f1* promoter provide cells, in which the initiation of epigenetic reprogramming can be observed in the living state. Such cells represent a strategic tool to answer a broad range of important questions in bovine development and embryo biotechnology, such as 1) reprogramming of different cell types after nuclear transfer, 2) the correlation between gene activation or silencing and changes in chromatin domain organization or nuclear architecture, and 3) the isolation of pluripotent stem cells.

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