Ovine iPSCs Are Resistant to Reprogramming after Nuclear Transfer

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Abstract

Induced pluripotent stem cells (iPSCs) share similar characteristics with ESCs of indefinite *in vitro* growth, and may therefore serve as a useful tool for the targeted genetic modification of farm animals via nuclear transfer (NT). Since derivation of stable ESC lines from farm animals has not been possible, it is important to determine whether iPSCs can be used as substitutes to ESCs for generating genetically modified cloned farm animals. We generated ovine iPSCs by conventional retroviral transduction using the four Yamanaka factors. These cells were bFGF- and Activin A-dependent, showed persistent expression of the transgenes, acquired chromosomal abnormalities and failed to activate endogenous *NANOG*. Nonetheless, iPSCs could differentiate into the three somatic germ layers *in vitro*. Since cloning of farm animals is best achieved with diploid cells (G_1/G_0), we synchronized the iPSCs in G1 prior to NT. Despite the cell cycle synchronization, preimplantation development of iPS-NT embryos was lower than with somatic cells (2% vs 10% blastocysts, $p< 0.01$). Furthermore, analysis of the blastocysts produced demonstrated persistent expression of the transgenes, aberrant expression of endogenous *SOX2*, and a failure to activate *NANOG* consistently. In contrast, gene expression in blastocysts produced with the parental foetal fibroblasts was similar to those generated by *in-vitro* fertilization. Taken together, our data suggest that the persistent expression of the exogenous factors and the acquisition of chromosomal abnormalities are incompatible with normal development of NT embryos produced with iPSCs.

Introduction

While embryonic stem cells (ESCs) can be derived from mouse and human blastocysts, ESCs from farm animals have not yet been established. Instead, cells with similar properties can be derived from fibroblast cells by forcing expression of exogenous reprogramming factors (Takahashi and Yamanaka, 2006) to generate induced pluripotent stem cells (iPSCs). Because iPSCs can self-renew long term in vitro and are amenable to complex genetic modification, they represent a valuable alternative for the generation of transgenic animals via nuclear transfer (NT).

Although iPSCs have been used to produce cloned mice by NT with equivalent success to ESC donors (Kou et al., 2010; Zhou et al., 2010), a large joint effort failed to produce cloned piglets using porcine iPSCs and successful term development was only obtained when iPSCs were differentiated before using them as NT donors (Fan et al., 2013). It is not clear what prevented postimplantation development of embryos reconstructed with undifferentiated iPSCs, but it is possible that either the lack of cell cycle synchronization prior to NT or the quality of the iPSCs compromised the developmental competence of those embryos. Pluripotent cells (ESCs, iPSCs and blastomeres) have a shortened overall cell cycle length with an extended S-phase. This means that using cycling cells as nuclear donors will result in poor development, because S-phase donor cells are incompatible with normal development after NT (Campbell and Alberio, 2003; Campbell et al., 1996a). Cloned farm animals are commonly produced using donor differentiated cells arrested in G_0/G_1 of the cell cycle (Campbell et al., 1996b; Kasinathan et al., 2001; Wells et al., 2003; Wilmut et al., 1997). Although some reports show that G2/M donors can also be used (Lai et al., 2002; Lai et al., 2001), other reports have found variable levels of aneuploidy and compromised development of the resulting reconstructed embryos (Alberio et al., 2000; Yuan et al., 2014; Zhang et al., 2004).

It is well accepted that NT with differentiated cells is very inefficient. In contrast, some studies have reported higher cloning efficiencies (live offspring) with mouse ESCs (Eggan et al., 2001; Jeanisch et al., 2002; Rideout et al., 2000; Wakayama et al., 1999). Despite higher development to term, the preimplantation development of ESC-reconstructed embryos is lower (15-29%) than embryos reconstructed with differentiated cells [40-67%, (Wakayama et al., 1998; Wakayama et al., 1999; Wakayama and Yanagimachi, 1999)]. It is possible that the lower preimplantation development of ESCs is due to lack of cell cycle synchronization between donor cells and recipient cytoplasts prior to NT.

In this study, we evaluated whether cell cycle synchronization into G_1 could improve preimplantation development of NT embryos using ovine iPSCs compared to parental fetal fibroblasts (PFFs). Reprogramming of key developmental genes (*NANOG*, *SOX2*, *CDX2* and *GATA3*) was analyzed in cloned blastocysts produced with iPSCs and PFFs as well as in control in-vitro fertilization (IVF) blastocysts. We found that reprogramming of *NANOG* and *SOX2* was abnormal in iPS-NT blastocysts compared to IVF and PFF-NT blastocysts. In addition, iPS-NT blastocysts showed persistent expression of the transgenes. Our data suggest that the quality of ovine iPSCs, rather than cell cycle synchronization, is the crucial factor determining normal embryo development after NT.

Materials and Methods

Generation of ovine iPSCs and culture of cells

The transfection of HEK (Human Embryonic Kidney) cells with individually packaged Oct4 (O), Sox2 (S), Klf4 (K) and c-Myc (M) viral vectors was performed as previously described (Breton et al., 2013). Forty-eight hours after transfection, supernatants (6 ml) were collected and filtered through 0.45 µm filters. Ovine parental foetal fibroblasts (PFFs; at passage four)

were seeded the day before onto a gelatine-coated 6-well plate at 2×10^5 cells/well and transduced with 1ml of each of OSKM virus supplemented with 4 µg/ml Polybrene (Sigma). The HEK cells were fed with 6ml fresh medium and the transduction was repeated 24 hours later. Forty-eight hours after first transduction, transduced PFFs were passaged using 0.25% trypsin-EDTA and seeded at 2×10^4 cells/well of a gelatine-coated 6-well feeder plate. Two days later, the culture medium was changed to iPSC medium [knock-out DMEM (Life Technologies) containing 20% knock-out serum replacement (Life Technologies), 2 mM Glutamax (Gibco), 0.1 mM non-essential amino acids (NEAA; Life Technologies), 0.1 mM 2-mercaptoethanol (Life Technologies) and supplemented with either 10 ng/ml human basic FGF (Peprotech)] and thereafter, the medium was changed daily. Transduced cells were passaged after 7 days and plated onto 6-well feeder plates at 5×10^3 cells per well. iPSC colonies were picked as they appeared and transferred to gelatine-coated 24-well feeder plates. iPSCs were split 1:2 every 2-3 days. For passaging the cells, 0.05% trypsin (Gibco) was used. iPSCs were incubated at 39°C, 5% CO2, 5% O2 in a humid incubator. PFFs were cultured in DMEM (Sigma) supplemented with 10% FBS, 2 mM Glutamax, 0.1 mM non-essential amino acids, 0.1 mM 2-mercaptoethanol and incubated at 39°C, 5% CO_2, 5% O_2. Mouse embryonic fibroblasts (MEFs) were cultured on 0.1% gelatine-coated flasks and maintained in DMEM supplemented with 10% FBS and 2 mM L-Glutamine. Low passage (<P4) MEF feeder cells were mitotically inactivated by 5 G ray gamma irradiation and seeded at 2×10^5 cells/well of a 6-well plate.

In-Vitro Differentiation

To test the ability of the clones to differentiate into the 3 germ layers (endoderm, ectoderm and mesoderm), we allowed the cells to form embryoid bodies (EBs) and then differentiate. iPSCs were passaged with collagenase and transferred onto bacterial grade non-adherent petri dishes without any feeder layer, fed with 2ml of fresh iPSC medium and incubated at 37°C

and 5% CO_2 overnight. The next day, the growing cells were transferred to a centrifuge tube and allowed to settle down by gravity for 20 minutes. The media was changed to 6 ml differentiation medium per tube (80% KO-DMEM, 10% FCS, 1x NEAA, 2mM L-Glutamine and 0.1 mM β-mercaptoethanol). Following the media change, the cells were then transferred to fresh petri dishes and incubated under the same conditions as before. The media was changed every two days until day 10. On day 11, EBs were transferred to two gelatin-coated wells of a 24-well tissue culture plate. The now differentiating EBs were fed with fresh media every day. On day 19, five wells of each plate were fixed with 4% paraformaldehyde (PFA). The cells were stored in 70% ethanol at 7°C for immunocytochemistry. Images illustrating EBs were recorded using a brightfield microscopy.

Colony formation assay

To assess self-renewal and proliferation abilities of iPSCs under different treatments, a colony formation assay was carried out. A 24-well plate (Nunc) was treated with 0.1% gelatine, MEFs were seeded at 6×10^4 cells/well and iPSCs were then seeded at 2000 cells/well. To block FGF receptors (FGFRs), MEK and ALK5 receptors, the following pharmacological inhibitors (from Tocris bioscience) were used: PD173074 (100 nM), PD0325901 (400 nM) and SB431542 (20 µM), respectively. The media was changed every day and cells were allowed to grow for ten days. Cells were then stained for alkaline phosphatase (AP) activity with the Leukocyte Alkaline Phosphatase Kit (Sigma) according to the manufacturer's instructions. Pictures were taken under bright field microscopy.

Immunofluorescence staining

Cells were fixed with 4% PFA and incubated with a blocking/permeabilization buffer (0.3% Triton-X, 10% bovine serum albumin and 10% goat serum in phosphate-buffered saline [PBS]). Cells were then incubated with primary antibody overnight at 4°C. Cells were then

rinsed three times in PBS and incubated with secondary antibody for 1 h at RT. After rinsing three times in PBS and counterstained with 300 ng/ml DAPI. A single wash was repeated and cells were left in PBS. Cells were viewed under confocal microscopy. The following antibodies were used: anti-cytokeratin (10 µg/ml, C2931, Sigma), anti-vimentin (10 µg/ml, V6630, Sigma), anti-beta III tubulin (10 µg/ml, MAB1637, Millipore), anti-OCT4 (1:100, sc-8628, Santa Cruz), anti-FGF2R (1:100, sc-122, Santa Cruz), anti-histone 3 phospho-serine 10 (1:500, ab32107, Abcam), anti-mouse IgG (R6393, Invitrogen) and anti-rabbit IgG (1:500, 711-025-152, Jackson Immunoresearch).

Cell cycle synchronization of iPSCs

To synchronize iPSCs, cultures were treated with 200 ng/ml of nocodazole (Sigma) for 16 h. Cells were then released by washing with PBS and replacing with growth medium. To assess the proportion of cells in S-phase (DNA synthesis) and M-phase prior and after synchronization, the cells were first stained with the Click-iT EdU Imaging Kit (Invitrogen) followed by immunostaining against the M-phase marker H3 phospho-serine 10, respectively. Briefly, iPSCs were given an EdU pulse of 30 min, trypsinized, washed and resuspended in PBS, transferred onto slides by cytospin at 190 rpm for 3 min, fixed in 4% PFA for 15 min at RT and stored in PBS at 4C. The cells were then stained for EdU according to the manufacturer's instructions and followed by immunofluorescence staining against the M-phase marker as described above.

Reverse-transcribed polymerase chain reaction (RT-PCR)

Total RNA was extracted from blastocysts and cells using the Absolutely RNA Nanoprep Kit (Agilent Technologies) and the RNeasy Mini Kit (Qiagen), respectively. Then, first strand cDNA was synthesized with SuperScript™ III Reverse Transcriptase (Invitrogen). The QIAGEN® Fast Cycling PCR kit was used to amplify target cDNA following the

manufacturer's instructions. Primers (table 1) were used at 300 nM. Amplification was carried out in a Mastercycler pro instrument (Eppendorf) with the following program: 45 cycles (35 cycles for GAPDH) at 96 °C for 10 sec, 60 °C for 30 sec, and 68 °C for 30 sec, with an initial activation step of 5 min at 95 °C and a final extension step at 72 °C for 1 min. The PCR products were resolved in 1.5% agarose gels.

Chromosome spreads

To increase the number of mitotic cells, demecolcine (Wako) was added to a final concentration of 0.1 ug/mL and incubated for 1 h. Cells were trypsinized, centrifuged at 250x g for 4 min, resuspended in 5 mL ice-cold 0.56% KCl (added dropwise while mixing) and incubated for 10 min at RT. Cells were then centrifuged and resuspended with 5 ml of ice-cold fixative (3 parts of methanol to 1 part of glacial acetic acid) added dropwise while vortexing at low speed. Cells were centrifuged, resuspended in 200 μL of fixative, dropped onto slides (pre-chilled at -20C) and air-dried. DNA was stained with Fluoroshield/DAPI mounting medium. Micrographs were taken with epifluorescence microscopy.

Oocyte maturation, NT and in vitro fertilization (IVF)

Ovine ovaries were collected from a local abattoir and transported to the laboratory in warm phosphate-buffered saline (PBS). Using needle and syringe, medium-sized (2-4 mm) follicles were aspirated. Cumulus-oocyte-complexes (COCs) with at least two layers of cumulus cells were selected for maturation in *in vitro* maturation (IVM) medium (bicarbonate buffered M199 medium [Gibco] supplemented with 10% heat-inactivated FBS, 0.05 U/ml FSH/LH [Pluset], 1.0 μg/ml 17β-oestradiol, 50 μg/ml gentamycin, 0.9 mM sodium pyruvate and 0.1 mM cysteamine). COCs were incubated in a humidified incubator at 39˚C and 5% CO_2 in air.

NT was performed as described previously (Choi et al., 2010; Lee et al., 2006). Briefly, oocytes were denuded of cumulus cells at 14 h of maturation by treatment with hyaluronidase

(0.9 mg/ml, Sigma) and gentle pipetting. Oocytes with extruding spindles at anaphase I/telophase I (AI/TI) were selected for enucleation. These oocytes were incubated in 10 µg/ml Hoechst 33342 and 7.5 µg/ml cytochalasin B in IVM medium for 5 min and enucleations were carried out in calcium-free HEPES buffered synthetic oviduct fluid (hSOF) with 7.5 µg/ml cytochalasin B using a micromanipulator. Enucleated oocytes were returned to IVM medium containing 10 mM caffeine for 6 h until cell transfer. Since serum starvation caused extensive cell death and differentiation in iPSCs, cells were first synchronized in M-phase with 200 ng/ml nocodazole for 16 h and then in G_1 by releasing the cells for 2 h in growth medium without nocodazole. Cells were then collected by trypsinization and resuspended in hSOF. The cells were kept at 4°C to slow down their metabolism and maintain cell cycle synchrony. PFFs were synchronized in G_0 by serum starvation for at least two days prior to NT. For cell transfer, one cell was placed into the perivitelline space of each enucleated oocyte and fused with one AC pulse of 1 V for 5 sec and two DC pulses of 100 V for 15 µs in fusion medium (0.3M mannitol, 0.1 mM magnesium sulfate, 280 mOsm). Reconstructed embryos were further cultured in IVM medium for 2 h. Embryos were then activated at 25-27 hpm in hSOF containing 5 µM calcium ionophore (A23187) and calcium ions for 5 min, rinsed and incubated in SOF medium (7.3 mM sodium pyruvate, 0.20 mM L-glutamine, 25 mM sodium bicarbonate, 5.4 mM sodium lactate, 108 mM sodium chloride, 7.2 mM potassium chloride, 1.2 mM potassium phosphate monobasic, 1.8 mM calcium chloride, 1.5 mM magnesium chloride, 0.34 mM tri-sodium-citrate, 2.8 mM myo-inositol, 10 µg/ml phenol red, 0.1 mM essential and non-essential amino acids, 4 mg/ml free-fatty-acids BSA, pH 7.4 and 270-290 mOsm) containing 10 ug/ml cycloheximide and 7.5 µg/ml cytochalasin B for 5 h at 39°C, 5% CO_2 and 5% O_2. Oocytes were then thoroughly rinsed and incubated in SOF. Medium was changed at days 2 and 5 after.

For IVF, ram semen was incubated in 3 ml capacitation medium (0.33 mM sodium pyruvate, 1.0 mM L-glutamine, 4.0 mM sodium bicarbonate, 21 mM HEPES, 3.35 mM sodium lactate, 108 mM sodium chloride, 7.2 mM potassium chloride, 1.2 mM potassium phosphate monobasic, 1.7 mM calcium chloride, 0.49 mM magnesium chloride, 2.4 mM glucose, 0.23 mM phenol red, 50 U/ml penicillin, 50 ng/ml streptomycin, pH 7.4 and 270-290 mOsm) for 1 h at 39 °C. Motile sperm was collected from the top 2 ml supernatant, centrifuged at 850g for 10 min at RT and resuspended in a small volume of IVF medium (2% FBS, 1.0 mM sodium pyruvate, 1.0 mM L-glutamine, 25 mM sodium bicarbonate, 10 mM sodium lactate, 108 mM sodium chloride, 7.2 mM potassium chloride, 1.2 mM potassium phosphate monobasic, 3.4 mM calcium chloride, 0.49 mM magnesium chloride, 1.4 mM phenol red, 50 U/ml penicillin, 50 ng/ml streptomycin, pH 7.9 and 270-290 mOsm). Matured COCs were incubated with 5 million sperm/ml in IVF medium for 20 h in a humidified incubator at 39˚C and 5% CO_2 in air. Presumptive zygotes were rinsed and cultured as described for NT.

Results

Characterization of bFGF/activin-dependent ovine iPSCs

The ovine iPSCs used in this study were generated by forced overexpression of the four Yamanaka factors (*Oct4, Sox2, c-Myc and Klf4*) in ovine foetal fibroblasts using retroviral transduction. These iPSCs displayed several characteristics associated with reprogramming towards pluripotency including formation of compact colonies in culture (Fig. 1A), downregulation of the fibroblast marker *FSP1* and upregulation of endogenous *SOX2* and *OCT4* (Fig.1 B), and positive AP staining (Fig. 1G). OCT4 was also detected by immunostaining together with FGFR2, a receptor for bFGF (Fig. 1C). Surprisingly, iPSCs were negative for *NANOG* (Fig. 1B). Expression of the trophectoderm (TE) markers *GATA3* and *CDX2* were not observed in either iPSCs or PFFs while they were detected in IVF

blastocysts (Fig. 1B). *COL1A1*, another gene highly expressed in fibroblasts, was also expressed in iPSCs (Fig. 1B), but expression levels were 70 times higher in the PFFs as estimated by quantitative RT-PCR (not shown). Ovine iPSC also expressed the exogenous reprogramming factors (Fig. 1D). At passage 40, metaphase spread analysis of iPSCs revealed that of the 50 spreads counted, all were diploid and 68% displayed a normal karyotype with 54 chromosomes (Fig. 1E), with the remaining 32% showed a range of aneuploidies (between 50 to 55 chromosomes). The parental cells had a normal karyotype.

Since bFGF was supplemented to the medium during the generation of these iPSCs and subsequent culture, we wished to determine whether the iPSCs required bFGF for self-renewal and proliferation. Furthermore, the cells were grown on a feeder layer of MEFs, a source of Activin A that helps iPSCs maintain pluripotency by signalling through ALK (Activin receptor-like kinase) receptors (Beattie et al., 2005). To study the FGF and Activin A requirements of these iPSCs, we carried out a colony formation assay by seeding the cells at low density and determined their AP activity after 10 days culture (Fig. 1G). Removing bFGF from the medium reduced the number of colonies (38%, total) as well as their average size (85%) relative to control (100%). Colony formation in bFGF-deprived medium was further decreased by inhibiting MEK signalling (24%) and still further with double inhibition of MEK and FGFRs (5%) relative to control. The size of bFGF-deprived iPSC colonies was progressively reduced by FGFR inhibition (70%), MEK inhibition (58%), ALK5 receptor inhibition (31%), FGF2R and MEK double inhibition (30%), and FGFRs and ALK5 double inhibition (20%) relative to control. We further confirmed the Activin A requirements of these iPSCs since they stained positive for the ectodermal marker beta III tubulin after inhibition of ALK5 receptors for 10 days (Fig. 1 F). Taken together, this assay showed that these ovine iPSCs required bFGF and that self-renewal and proliferation were regulated by FGF/ERK together with Activin A signalling pathways.

To test whether these ovine iPSCs were pluripotent, we assessed their capacity to differentiate in vitro into cell lineages derived from the three somatic germ layers. Under conditions inducing differentiation (see Materials and Methods), the cells aggregated into embryoid bodies and, after plating, they differentiated into cells positive for alpha-fetoprotein (endoderm), vimentin (mesoderm) and beta III tubulin (ectoderm) (Fig. 2).

Cell cycle synchronization of ovine iPSCs

We used EDU labelling and anti phospho-histone H3 immunostaining to determine the cell cycle profile of iPSCs. In continuous iPSCs cultures 40% of cells were in S-phase while 5% were in mitosis, with the remaining 55% in G_1 and G_2. We first attempted to carry out NT with cells that had been synchronized in M phase by a short block with demecolcine followed by 'shake off' to release the mitotic cells (Kasinathan et al., 2001). This preliminary data showed that development to blastocysts with M phase donors was very low, 4% for PFF-NT and 0% for iPS-NT. Therefore, we opted to synchronize the iPSCs in G_1 using nocodazole, since synchronisation by serum starvation to G1/G0 resulted in extensive cell death and differentiation. To this end, we synchronized the cells in M-phase by nocodazole block for 16 h and then released them (Fig. 3A-B). After 16 h block with 200 ng/ml of nocodazole, the proportion of iPSCs in mitosis was enriched to 55%, while the proportion of cells in S-phase was halved (20%). After 2 h release from nocodazole, these mitotic cells had progressed into G_1 since only 4% of cells remained in M and 9% were in S. iPSCs entered S-phase between 4 h and 6 h post-release, with about 66% of cells progressing from G1 to S phase between 4 h and 10 h post-release (Fig. 3A-B). These data show that the proportion of ovine iPSCs in G_1 can be enriched by nocodazole block and release. After 2 h release, we selected small iPSCs as presumptive G_1 donors for NT (Fig. 3C).

Ovine iPSCs produce fewer blastocysts than PFFs after NT

After synchronization of iPSCs in G_1 and of PFFs in G_0 prior to NT, we determined the preimplantation development of the reconstructed embryos (table 2). Results from three replicates show that cleavage rate was high in both groups (79% for iPS-NT and 90% for PFF-NT), however blastocyst formation was significantly lower with iPS-NT (2%) compared to PFF-NT (10%, $p < 0.01$, Chi square analysis).

iPSCs are not fully reprogrammed following NT

To assess nuclear reprogramming in reconstructed embryos with different donor cells, we analysed the expression of *NANOG*, *GATA3* and *CDX2* at the blastocyst stage by endpoint RT-PCR. We also included *SOX2* in the analysis to determine whether this gene, which is highly expressed gene in iPSCs, was downregulated in iPS-NT blastocysts. Analysis was carried out in three biological replicates for both groups together with IVF controls (Fig. 4A). We did not detect *SOX2* in any of the IVF blastocysts, while expression was observed in 2/3 of replicates in iPS-NT blastocysts and 1/3 in PFF-NT blastocysts (Fig. 4B-C). The TE markers *GATA3* and *CDX2* were consistently activated in all replicates (Fig. 4B). Surprisingly, *NANOG* was not activated in 2/3 iPS-NT blastocysts, while IVF and PFF-NT blastocysts activated this pluripotency gene in all three replicates (Fig. 4B-C).

We also investigated whether the exogenous pluripotency genes expressed in iPSCs were silenced following NT. We found that all four exogenous genes were still expressed both at the blastocyst stage and in embryos that failed to form blastocysts (Fig. 4D).

Discussion

In this study we investigated the growth characteristics and developmental capacity of bFGF-dependent ovine iPSCs. We confirmed the dependency of these cells on bFGF and Activin A

signalling for self-renewal and proliferation, similar to the signalling requirement of hESC and epiblast stem cells (Alberio et al., 2010; Brons et al., 2007; Tesar et al., 2007; Vallier et al., 2005). However, in contrast to human ESCs and epiblasts stem cells, which form flat colonies and usually do not tolerate passaging by trypsin, our ovine iPSCs formed dome-shaped colonies and were passaged as single-cell suspensions. In this respect, ovine iPSCs were more similar to mouse ESCs. These characteristics are consistent with ovine iPSCs reported in previous studies (Bao et al., 2011; Liu et al., 2012; Sartori et al., 2012).

Nonetheless, using a colony formation assay, we showed that our ovine iPSCs were bFGF and Activin A dependent. Removing bFGF from the culture medium resulted in fewer and smaller colonies, and this was further confirmed using FGF/ERK signalling inhibitors. Furthermore, inactivation of the ALK5 receptors with a specific inhibitor had a more pronounced effect at reducing proliferation. This is consistent with previous reports showing that the Activin A/Nodal pathway is required to maintain self-renewal of mouse epiblast stem cells (Brons et al., 2007; Tesar et al., 2007) as well as pluripotency in pig epiblast stem cells (Alberio et al., 2010; Park et al., 2013). Double inhibition of FGFRs and MEK or FGFRs and ALK5 had a synergistic effect on proliferation compared to either inhibitor alone. Taken together, these functional data support that both FGF/ERK and Activin A transduction signalling pathways are important for self-renewal of ovine iPSCs.

Persistent expression of transgenes has been reported in iPSC lines from pigs (Esteban et al., 2009; Ezashi et al., 2009; Fan et al., 2013; Rodriguez et al., 2012; West et al., 2010), cattle (Sumer et al., 2011), mice (Okita et al., 2007), humans (Liu et al., 2009; Ramos-Mejia et al., 2012), monkeys (Liu et al., 2008) and sheep (Sartori et al., 2012). Moreover, it appears that overexpression of the transgenes is necessary to maintain pluripotency in iPSCs of farm animals since differentiation has been reported following inactivation of the exogenous

factors in doxycycline (DOX) inducible systems (Bao et al., 2011; Hall et al., 2012; Li et al., 2011, Rodriguez et al., 2012).

This continued dependency on expression of exogenous reprogramming factors suggests incomplete activation of the network of endogenous genes associated with pluripotency. Although both ovine studies involving DOX-inducible systems reported expression of endogenous *OCT4*, *SOX2* and *NANOG* (Bao et al., 2011; Li et al., 2011), it is possible that other genes associated with pluripotency were not activated or that expression levels of endogenous pluripotent genes were low and thus insufficient to maintain pluripotency without expression of the transgenes. We found that endogenous *OCT4* and *SOX2* were expressed while *NANOG* was not, suggesting incomplete reprogramming of the pluripotency gene network. The lack of *NANOG* expression was consistent in multiple iPSC lines generated (not shown). Although *NANOG* is essential for pluripotency in the embryo (Chambers et al., 2003), recent evidence shows that iPSCs with full differentiation capacity can be generated without *Nanog* (Carter et al., 2014; Schwarz et al., 2014), suggesting that the four-factor overexpression system can bypass the requirement of Nanog during iPS reprogramming. In agreement with these reports, ovine iPSCs were capable of differentiating into cells derived from the three germ layers in vitro, demonstrating that they were pluripotent. However, we found that *NANOG* reactivation was very limited in iPS cloned blastocysts, suggesting that these iPSCs were locked in a reprogrammed state resistant to the reprogramming machinery of the oocyte.

Despite the synchronization of iPSCs in G_1, we found that development to blastocysts of cloned embryos was not improved compared to those produced using fibroblasts. This finding is consistent with a recent publication reporting similar developmental competence of pig iPSCs and fibroblasts (Xie et al., 2014). While this manuscript was in preparation, a report showed that iPSCs synchronized in G_2/M are better suited for NT compared to G_1 and

unsynchronized cells (Yuan et al., 2014). Improved development with G_2/M synchronized donors was also reported with mouse iPSCs (Kou et al., 2010). However, the general observation with G2/M phase donors is that the resulting embryos have increased proportion of aneuploidies, therefore, unlikely to support full term development.

We next sought to determine features of genetic reprogramming in cloned embryos produced with different cell types. We found that blastocysts produced with PFFs are better reprogrammed than those produced with iPSCs. *NANOG*, which was silent in both iPSCs and PFFs, remained inactive in most iPS-NT blastocysts, while it was consistently expressed in PFF-NT and IVF blastocysts. This was surprising since it was expected that a gene associated with pluripotency would be activated more easily in iPSCs than in differentiated cells. *SOX2*, in contrast, was not expressed in any of the IVF blastocysts, whereas it was expressed in 2/3 iPS-NT blastocysts and in 1/3 of PFF-NT blastocysts. This suggests that *SOX2* was not properly silenced following iPS-NT. It was surprising not to detect activation of *SOX2* in IVF blastocysts since we had previously detected low levels in control IVF blastocysts when characterising gene expression of the cells (Fig. 1). One possibility for this incongruence is that *SOX2* starts to be expressed at this stage at low levels in some blastocysts, so it could be detected in a pool of ten blastocysts (Fig. 1) but not in individual blastocysts (Fig. 4). Alternatively, culture conditions might have affected gene expression as the IVF control embryos used in Fig. 1 had been cultured with BSA while the ones in Fig. 4 were cultured with FBS (as were the cloned embryos). As with endogenous *SOX2*, we observed no silencing of exogenous pluripotency genes during preimplantation development of sheep iPS-NT embryos, which is consistent with the recent publication in pigs (Xie et al., 2014). Activation of trophectoderm lineage markers was normal as both iPS-NT and PPF-NT blastocysts expressed *CDX2* and *GATA3*. This is consistent with a previous study in mice, which showed CDX2 expression in the trophectoderm of iPS-NT blastocysts by

immunocytochemistry (Kou et al., 2010). With the exception of the trophectoderm lineage, our data support that genetic reprogramming in iPSCs is incomplete following NT. The incomplete reprogramming of iPSCs after NT attests to the poor quality of iPS-NT embryos and could explain the low development of cloned iPSCs embryos reported here and in a previous study (Fan et al., 2013). To our knowledge, this is the first study comparing gene expression reprogramming of key developmental genes between cloned blastocysts produced with iPSCs versus parental fibroblast cells.

In summary, our data support that reprogramming of ovine iPSCs after NT is incomplete. This might be an important factor explaining the low developmental capacity of reconstructed embryos with iPSCs in farm animals. Persistent expression of transgenes and increased number of aneuploidies in donor cells may be the reason for compromised reprogramming and developmental potential NT embryos, although the underlying reason for these has yet to be determined. Nonetheless, from our results in sheep and from those reported by other groups in pigs (Fan et al., 2013; Xie et al., 2014), it is plausible to propose that iPSCs generated by non-integrating methods (free of transgenes) would be better candidates for the production of transgenic animals by NT.

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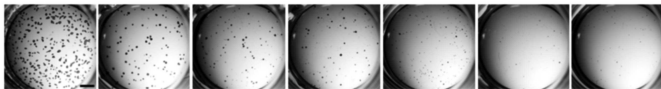
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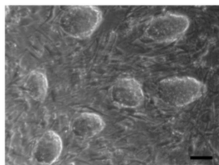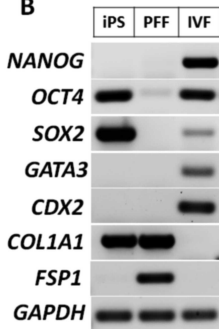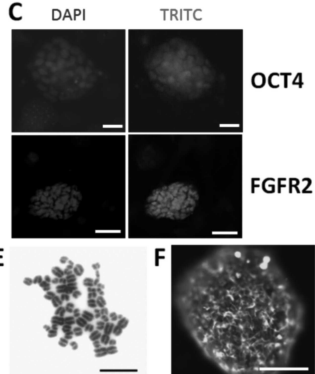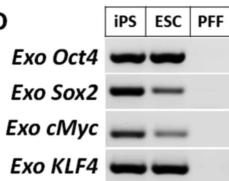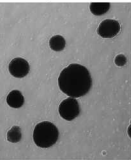
bFGF	+	-	-	-	-	-	-
FGFR i	-	-	+	-	-	+	+
MEK i	-	-	-	+	-	+	+
ALK5 i	-	-	-	-	+	-	+
No. colonies	289	109	102	70	151	15	65
Colony size (µm)	261 ± 58	222 ± 56	183 ± 58	151 ± 52	81 ± 31	77 ± 29	52 ± 15

Embryoid bodies	Endoderm	Mesoderm	Ectoderm
	Alpha-fetoprotein	Vimentin	Beta III tubulin
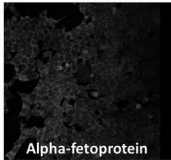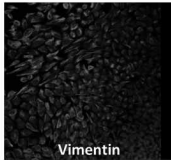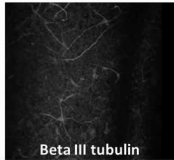
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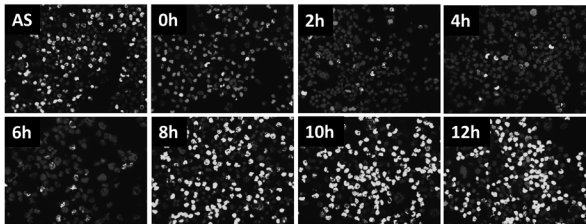
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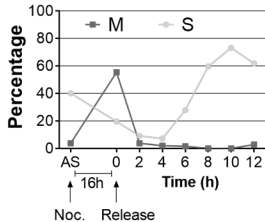
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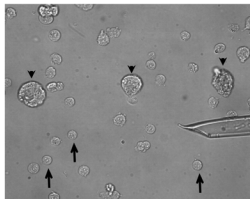
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| Replicate No. | 1 | 2 | 3 |

PFF-NT

iPS-NT

IVF
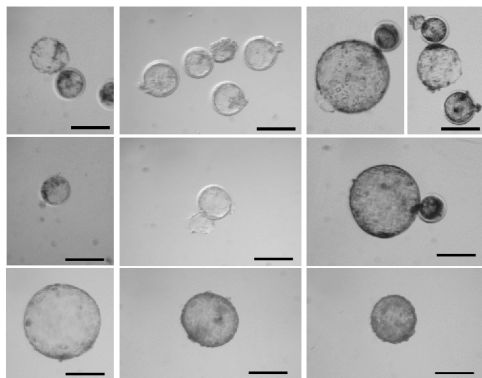
B

Blastocyst	SOX2	NANOG	GATA3	CDX2
IVF	0/3	3/3	3/3	3/3
iPS-NT	2/3	1/3	3/3	3/3
PFF-NT	1/3	3/3	3/3	3/3

C

	IVF	iPS-NT	PFF-NT
SOX2			
NANOG			
GATA3			
CDX2			
GAPDH			

D

	Oct4	C-Myc	KLF4	Sox2	GAPDH
iPSCs					
iPS blast #2					
iPS blast #3					
iPS arrested embryos					

Exogenous genes