Activation of bovine somatic cell nuclear transfer embryos by PLCZ cRNA injection

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Abstract

The production of cloned animals by the transfer of a differentiated somatic cell into an enucleated oocyte circumvents fertilization. During fertilization, the sperm delivers a sperm-specific phospholipase C (PLCZ) that is responsible for triggering Ca^{2+} oscillations and oocyte activation. During bovine somatic cell nuclear transfer (SCNT), oocyte activation is artificially achieved by combined chemical treatments that induce a monotonic rise in intracellular Ca^{2+} and inhibit either phosphorylation or protein synthesis. In this study, we tested the hypothesis that activation of bovine nuclear transfer embryos by PLCZ improves nuclear reprogramming. Injection of PLCZ cRNA into bovine SCNT units induced Ca^{2+} oscillations similar to those observed after fertilization and supported high rates of blastocyst development similar to that seen in embryos produced by IVF. Furthermore, gene expression analysis at the eight-cell and blastocyst stages revealed a similar expression pattern for a number of genes in both groups of embryos. Lastly, levels of trimethylated lysine 27 at histone H3 in blastocysts were higher in bovine nuclear transfer embryos activated using cycloheximide and 6-dimethylaminopurine (DMAP) than in those activated using PLCZ or derived from IVF. These results demonstrate that exogenous PLCZ can be used to activate bovine SCNT-derived embryos and support the hypothesis that a fertilization-like activation response can enhance some aspects of nuclear reprogramming.

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Introduction

The generation of live offspring after somatic cell nuclear transfer (SCNT) has been successfully achieved in numerous mammalian species (Cibelli 2007). However, the overall efficiency of the technology remains extremely low (Wilmut *et al.* 2002). Failure to correctly reprogram the somatic cell genome following transfer into the oocyte cytoplasm has been hypothesized to be a major cause of developmental abnormalities (Shi *et al.* 2003, Hochedlinger & Jaenisch 2006). Nuclear reprogramming – the process by which a specialized nucleus reacquires developmental potential, adopting the role of a zygotic nucleus – involves silencing somatic-specific genes and activating essential embryonic genes (Latham 2005). Although the process of nuclear reprogramming is not fully understood, it is becoming evident that epigenetic modifications of chromatin (e.g. methylation and/or acetylation of DNA and histones) are fundamental for the regulation of gene expression (Reik *et al.* 2001, Rideout *et al.* 2001).

In most mammalian species, oocytes are ovulated at the MII stage of meiosis and remain arrested until

fertilized by sperm. Initiation of development is triggered by a series of long-lasting oscillations of intracellular free calcium ($[\text{Ca}^{2+}]_i$). Several pieces of evidence support the hypothesis that the sperm, upon fusion with the oocyte, delivers a sperm-specific isoform of phospholipase C (PLCZ; Saunders *et al.* 2002, Kurokawa *et al.* 2004, Swann *et al.* 2004, 2006, Knott *et al.* 2005, Malcuit *et al.* 2006a). PLCZ has the ability to function at basal Ca^{2+} concentrations (Kouchi *et al.* 2004) and thus, upon entering the oocyte's cytoplasm, induces hydrolysis of phosphatidylinositol-4, 5-bisphosphate (PIP_2), generating 1, 2-diacylglycerol (DAG), and IP_3 . In turn, IP_3 binds to its receptor (IP_3R), located on the endoplasmic reticulum membrane, and induces a conformational change that allows release of Ca^{2+} into the oocyte's cytoplasm. By an as yet uncharacterized mechanism, Ca^{2+} release and uptake are repeated, generating what is described as $[\text{Ca}^{2+}]_i$ oscillations. The pattern of $[\text{Ca}^{2+}]_i$ oscillations at fertilization is species specific, and these oscillations have been observed to occur over a relatively long period of time (3 to 4 h in mice; 16–18 h in cattle).

The significance of the $[Ca^{2+}]_i$ oscillatory pattern has been well studied in mice and rabbits (Ozil & Huneau 2001, Ducibella *et al.* 2006). Alterations in oocyte calcium signaling affected not only the early events of embryonic development (Ducibella *et al.* 2002, Ozil *et al.* 2005), but also gene expression in eight cell (Rogers *et al.* 2006) and blastocyst-stage embryos (Ozil *et al.* 2006), implantation (Ozil & Huneau 2001, Ozil *et al.* 2006), and even development to term (Ozil *et al.* 2006). It is possible that the long-lasting effect of $[Ca^{2+}]_i$ oscillations was mediated by alterations in chromatin structure and reprogramming of gene expression occurring after fertilization (Ozil & Huneau 2001, Ozil *et al.* 2006, Rogers *et al.* 2006). These observations suggest that improper oocyte activation may affect the level of nuclear reprogramming following SCNT.

Most activation protocols commonly used during SCNT rely on chemicals that not only induce a non-physiological Ca^{2+} transient pattern, but also affect other cellular processes, with possible negative consequences for embryonic development (Alberio *et al.* 2001a, 2001b). For example, the use of cycloheximide (CHX) during activation of bovine SCNT embryos was associated with delayed DNA synthesis (Alberio *et al.* 2001b), and the use of 6-dimethylaminopurine (DMAP) as the activating agent often resulted in a high proportion of aneuploid embryos (Winger *et al.* 1997, Van De Velde *et al.* 1999, Bhak *et al.* 2006).

We have previously shown that PLCZ cRNA injection into bovine oocytes was able to induce long-lasting $[Ca^{2+}]_i$ oscillations and IP₃R-1 downregulation and that it supported high rates of parthenogenetic development to the blastocyst stage (Malcuit *et al.* 2006b, Ross *et al.* 2008b). In the present study, we compared *in vitro* development, gene expression patterns, and epigenetic

modifications in SCNT embryos activated by PLCZ cRNA injection with embryos activated by chemical methods or produced by IVF. Although all activation protocols supported oocyte activation and embryo development, embryos activated by PLCZ displayed gene expression profiles and levels of trimethylated lysine 27 at histone H3 (H3K27me3) that closely resembled those seen in IVF embryos.

Results

PLCZ triggers fertilization-like $[Ca^{2+}]_i$ oscillations in bovine oocytes reconstructed by SCNT

We have previously shown that injection of PLCZ cRNA into bovine oocytes induced long-lasting $[Ca^{2+}]_i$ oscillations, downregulated IP₃R-1, and supported parthenogenetic development to the blastocyst stage (Malcuit *et al.* 2006b, Ross *et al.* 2008b). In the present study, we extended these findings and confirmed that PLCZ cRNA injection triggers fertilization-like $[Ca^{2+}]_i$ oscillations in oocytes reconstructed by SCNT (Fig. 1). During the first 3 h after injection, a series of $[Ca^{2+}]_i$ oscillations were observed with intervals of 28 min, and then their frequency increased to one oscillation every 8 min from 5 to 9 h after PLCZ injection (Table 1). Some oocytes (5 out of 10) stopped oscillating during the recorded period at 9, 10.5, 11, 12, and 13 h after PLCZ injection. For those in which oscillations continued, the frequency started to decrease, with oscillations occurring every 26 min from 11 to 14 h post-activation.

The ability to induce repetitive $[Ca^{2+}]_i$ rises by PLCZ cRNA injection provided a method to mimic the sperm-induced oocyte activation stimulus and to test the hypothesis that a more physiological oocyte activation stimulus can improve reprogramming after SCNT.

Figure 1 Representative $[Ca^{2+}]_i$ profiles observed in SCNT embryos from 1 to 14 h after murine PLCZ cRNA injection.

Table 1 Time interval between $[Ca^{2+}]_i$ oscillations in SCNT embryos activated using PLCZ cRNA injection.

Time after PLCZ injection (h)	Oscillating oocytes (n)	Mean $\pm$ S.E.M. interval between $[Ca^{2+}]_i$ increases (min)
1 to 3	10/10	28.4 $\pm$ 1.6
3 to 5	10/10	13.2 $\pm$ 2.6
5 to 7	10/10	8.1 $\pm$ 1.3
7 to 9	10/10	8.1 $\pm$ 2.1
9 to 11	9/10	19.8 $\pm$ 5.3
11 to 14	5/10	25.7 $\pm$ 6.3

Embryonic development of cloned embryos activated by PLCZ cRNA injection

To evaluate whether PLCZ cRNA injection supports activation and *in vitro* development of bovine SCNT embryos, we injected mPLCZ cRNA into oocytes reconstructed by nuclear transfer and compared cleavage and development rates induced by this treatment with those induced by chemical activation and by IVF. Table 2 presents the results, and representative figures of blastocysts obtained with the different activation protocols are shown in Fig. 2. A total of 769 bovine SCNT embryos were produced in nine replicates. Equivalent cleavage rates, ranging from 75.4 to 79.2% ($P > 0.05$), were observed among SCNT groups. The cleavage rate (87.0%) of IVF embryos was higher than that of SCNT embryos ($P < 0.05$). Development of the embryos to blastocyst stage was the highest for SCNT embryos activated with ionomycin/CHX and the lowest for embryos produced by IVF ($P < 0.05$).

As indicators of embryo quality, we determined the total number of cells and their allocation to the inner cell mass (ICM) and trophectoderm (TE) of expanded/hatched blastocysts. No differences were observed in total, TE or ICM cell numbers (Fig. 3a and c); however, the ratio of ICM:TE cells was higher in SCNT embryos activated using DMAP than in those activated using CHX or derived from IVF ($P < 0.05$). PLCZ-activated embryos did not differ from IVF or SCNT groups.

We also determined the incidence of apoptosis among blastocysts produced with the different activation strategies. The proportion of apoptotic cells in blastocysts, as assessed by TUNEL staining, was similar among all of the groups analyzed ($P > 0.05$; Fig. 3a and b).

Table 2 Preimplantation development of IVF- and SCNT-derived embryos activated using different protocols.

Treatment	Embryos cultured	Cleaved (%)	Blastocysts (%)
IVF	492	428 (87.0) ^a	125 (25.4) ^a
PLCZ	332	262 (78.9) ^b	92 (27.7) ^{a,b}
Iono/DMAP	327	259 (79.2) ^b	109 (33.3) ^{b,c}
Iono/CHX	329	248 (75.4) ^b	118 (35.9) ^c

^{a,b,c} Percentages not sharing a common letter are statistically different ($P < 0.05$, χ^2 -test).

Finally, we performed chromosomal analysis at the blastocyst stage, since maintenance of normal ploidy is a prerequisite for embryos to develop to term. The fibroblast donor cell line used in this study had a normal diploid chromosomal composition. After SCNT, we observed that 86% of the embryos were diploid at the blastocyst stage irrespective of the activation protocol and were similar to IVF-derived embryos (Table 3).

Taken together, these results indicate that PLCZ cRNA injection was an effective method to induce activation of embryonic development after SCNT and that the embryos generated by this method presented similar characteristics to embryos produced by IVF in terms of cell number and allocation, apoptosis and embryo ploidy.

Effect of oocyte activation method on gene expression at 8-cell and blastocyst stages

Reprogramming of gene expression involves reactivation of important embryonic genes as well as the repression of somatic cell-specific genes. To test whether the type of activation stimulus affects the reactivation of embryonic genes, we first evaluated the abundance of transcripts at the eight-cell stage, which is the earliest stage where embryonic genome activation can be consistently assessed in bovine embryos. The expression levels of these transcripts were normalized to an external control to account for differences in RNA extraction and RT efficiency.

Gene expression analysis at the eight-cell stage revealed that cloned embryos were able to express desmocollin 2 (*DSC2*) and glucose transporter 1 (*GLUT1*) at levels similar to IVF embryos ($P > 0.05$; Fig. 4), although *GLUT1* expression was affected by the type of SCNT activation protocol, with embryos activated using CHX presenting a higher level of transcripts than those activated by PLCZ cRNA injection ($P < 0.05$). On the other hand, *POU5F1* (Oct-3, OCT4) transcript abundance was significantly lower in DMAP-activated SCNT embryos than in IVF-derived embryos.

We also performed gene expression analysis at the blastocyst stage to evaluate expression of genes that are characteristic of the two cell lineages that comprise the blastocyst. *POU5F1*, *NANOG*, and *SOX2* have been characterized as important for pluripotency and ICM formation, while the transcription factor *CDX2* and the FGF receptor type 2 (*FGFR2*) have been shown to be expressed specifically in the TE of mouse embryos. We also determined the expression of *TRYP8*, a gene expressed by somatic cells but not during pre-implantation development of fertilized embryos, and of *U2AF1L2*, a gene that in a previous study (Beyhan *et al.* 2007) we found discriminated between IVF and SCNT embryos. Blastocyst gene expression was normalized to the exogenous control and then to the total cell number in the embryo, as determined just

Figure 2 Representative pictures of blastocysts generated by IVF and SCNT using different activation protocols.

before embryo lysis. We found that *GAPDH*, *POU5F1*, and *CDX2* were expressed at significantly lower levels in CHX-activated SCNT embryos than in the other groups (Fig. 5). *U2AF1L2*, a gene involved in RNA splicing, was only detected in IVF embryos. Finally, *TRYP8* that was expressed at high levels in the donor cells, was amplified in a higher proportion ($P < 0.05$) of SCNT embryos activated by CHX and DMAP (60 and 62.5% respectively) than in IVF and SCNT embryos activated using PLCZ (11 and 33% respectively).

Chromatin modifications in embryos produced by IVF or SCNT and activated by different means

Although, the precise mechanism of nuclear reprogramming after SCNT has not been elucidated, chromatin remodeling is known to play a fundamental role. Chromatin remodeling involves, among other modifications, changes in acetylation and methylation of histone tails. In the present study, we used immunofluorescence to evaluate genome-wide histone methylation at histone H3 lysine 27, a change that is associated with gene

silencing and histone acetylation at histone H4 lysine 5, a modification that is associated with transcriptional activation (Fig. 6). We found that the levels of acetylated histone did not differ among groups (Fig. 6b); however, the levels of H3K27me3 were higher in bovine SCNT embryos activated using CHX or DMAP compared with those activated using PLCZ or derived from IVF ($P < 0.05$; Fig. 6c).

Discussion

SCNT remains an inefficient technique in spite of almost 10 years of research since the first mammal was cloned (Cibelli 2007). This inefficiency is commonly attributed to incomplete reprogramming of the somatic cell nucleus (Reik *et al.* 2001, Rideout *et al.* 2001, Latham 2004, Cibelli *et al.* 2006). Nuclear reprogramming is initiated by the recipient oocyte and likely continues through early embryonic development (Latham 2004). Oocyte activation may influence the reprogramming ability of the oocyte. Alterations of the signaling mechanism leading to oocyte activation and initiation

Figure 3 Cell number, allocation, and apoptosis in IVF and SCNT embryos produced using different activation methods. (a) Representative images of analyzed embryos; ICM (blue), TE (red), and TUNEL-positive nuclei (green). (b) Quantification of TUNEL-positive cells per embryo. (c) Comparison of cell number and allocation among groups. ^{a,b} bars not sharing a common letter are statistically different ($P < 0.05$).

Table 3 Chromosomal composition of blastocysts activated using different protocols.

Treatment	n	Diploid	Tetraploid	Mixoploid	Total abnormal
IVF	15	13	2		2 (13.3%)
PLCZ	12	10	1	1 ^a	2 (16.7%)
Iono/DMAP	12	11	1		1 (8.3%)
Iono/CHX	20	17	2	1 ^b	3 (15.0%)

^aDiploid/triploid. ^bDiploid/tetraploid.

of embryonic development was shown to affect early events of oocyte activation – like mRNA translation (Ducibella *et al.* 2002, 2006) – as well as fetal and term development (Ozil *et al.* 2006), probably by affecting the reprogramming of the zygotic genome. In all mammals studied so far, oocyte activation is characterized by long-lasting $[Ca^{2+}]_i$ oscillations, which persist for several hours (Malcuit *et al.* 2006a). During bovine SCNT, oocytes are usually activated using chemicals that induce a single increase in $[Ca^{2+}]_i$, supplemented with broad-spectrum protein synthesis inhibitors or kinase inhibitors (Alberio *et al.* 2001a, Machaty 2006, Malcuit & Fissore 2007). These treatments that do not recapitulate the signaling events triggered by the fertilizing sperm may have adverse consequences for nuclear reprogramming and for the development of cloned embryos (Alberio *et al.* 2001a, Malcuit & Fissore 2007). We hypothesized that activating bovine SCNT embryos with a system that closely mimics the activation mechanism brought about by the sperm would result in a more efficient nuclear reprogramming. To address this hypothesis, we implemented a sperm-like activation protocol to induce activation of SCNT embryos.

PLCZ that has been characterized in mice (Saunders *et al.* 2002), humans (Rogers *et al.* 2004), pigs (Yoneda *et al.* 2006), and cattle (Ross *et al.* 2008b), is believed to be the activation factor that the sperm delivers upon fusion with the oocyte at fertilization. After injecting mPLCZ cRNA into SCNT-reconstructed bovine embryos, we observed long lasting $[Ca^{2+}]_i$ oscillations similar to those elicited by fertilization (Fissore *et al.* 1992, Nakada *et al.* 1995); however, the increased frequency of $[Ca^{2+}]_i$ oscillations seen in SCNT embryos between 5 and 9 h after PLCZ injection was not observed after IVF; which likely reflects protein accumulation with increased

translation time. Along with our previous observations – that PLCZ cRNA injection into MII oocytes induced $[Ca^{2+}]_i$ oscillations, IP₃R-1 downregulation, and parthenogenetic development (Ross *et al.* 2008b) – these data suggest that injection of PLCZ cRNA into oocytes reconstructed by SCNT can be used as an activation stimulus that mimics sperm-induced activation.

PLCZ was effective at inducing pre-implantation development of SCNT embryos, which did not seem to be affected by the type of activation stimulus. SCNT embryos activated by PLCZ cRNA injection or common chemical activation stimuli (ionomycin/DMAP and ionomycin/CHX) produced blastocysts at rates of 25 to 36%, which are comparable with those reported in the literature for IVF and SCNT embryos (Cibelli *et al.* 1998, Zakhartchenko *et al.* 1999, Heyman *et al.* 2002, Kane 2003). Total cell number and number of cells allocated to the TE and ICM have been regarded as valuable indicators of cattle embryo quality (van Soom *et al.* 1997, 2001). A higher total cell number in the embryo correlated with IVF (van Soom *et al.* 1997) and SCNT embryos' developmental potential (Renard *et al.* 2007). Moreover, it has been suggested that aberrant allocation of ICM and TE cells to the blastocyst stage may be responsible for the abnormalities observed after transfer of SCNT embryos (Koo *et al.* 2002). Like other observers, we found that SCNT embryos activated using ionomycin/DMAP presented a significantly higher ratio of ICM:total cells when compared with IVF and *in vivo*-produced embryos (Koo *et al.* 2002). On the other hand, we saw no differences between embryos activated using ionomycin/CHX or PLCZ cRNA injection and IVF controls, which

Figure 4 Quantification of mRNA abundance in eight-cell embryos generated by IVF or SCNT using different activation protocols. ^{a,b}: bars not sharing a common letter are statistically different ($P < 0.05$).

Figure 5 Quantification of mRNA abundance in blastocysts generated by IVF or SCNT using different activation protocols. ^{a,b}: bars with different superscripts differ significantly ($P < 0.05$).

Figure 6 Histone methylation and acetylation analysis of IVF and SCNT embryos activated using different protocols. (a) Representative pictures of immunostained blastocyst-stage embryos. (b) Semiquantitative evaluation of H4K5Ac and (c) H3K27me3 immunostaining. ^{a,b}: bars with different superscripts differ significantly ($P < 0.05$).

suggests that activating SCNT embryos by these means may improve their developmental potential.

When we looked at reactivation of embryonic genes in cloned embryos, PLCZ presented a similar pattern of gene expression to IVF controls, while ionomycin/CHX- and ionomycin/DMAP-activated embryos showed some differences compared with IVF embryos. For example, a housekeeping gene (*GAPDH*), a transcription factor important for ICM development (*POU5F1*), and a transcription factor important for TE development (*CDX2*) were expressed at lower levels in SCNT embryos activated by ionomycin/CHX compared with the other groups. These abnormalities in gene expression were in all cases observed for only one of the chemical treatments. This led us to speculate that they were a consequence of the inhibition of protein synthesis (CHX) or protein phosphorylation (DMAP), but not because of the abnormal $[Ca^{2+}]_i$ regimen. On the other hand, in agreement with our previous report (Beyhan *et al.* 2007), *U2AF1L2*, a gene involved in RNA splicing, was only detected in IVF embryos, indicating that reprogramming of this locus failed in SCNT embryos irrespective of the activation protocol. Although the role of *U2AF1L2* in embryonic development has not been investigated, the intrinsic capacity of this gene to affect several cellular processes could lead

to potentially serious alterations in the ability of cloned embryos to develop normally. Furthermore, this suggests that some abnormalities associated with SCNT are not improved by activation with PLCZ.

Activation with PLCZ required treatment with cytochalasin B (CB) to avoid the segregation of a second polar body containing part of the somatic cell chromosomes (Supplementary Figure 1, which can be viewed online at www.reproduction-online.org/supplemental/). CB is an actin-polymerization inhibitor that may detrimentally affect embryonic development or nuclear reprogramming by not allowing the movement of intracellular molecules to their required sites of action. Also, a different $[Ca^{2+}]_i$ oscillatory pattern may be necessary to induce reprogramming of a somatic cell nucleus rather than to reprogram the gametes' chromatin, e.g., during fertilization. Moreover, other components of the sperm that are not provided by the somatic cell may be required to initiate normal embryonic development. For instance, the sperm protein PAWP (WBP2NL) that resides in the perinuclear theca was found to be required for meiotic resumption and pronuclear development following fertilization (Wu *et al.* 2007). Also, mature sperm carried full length mRNAs that were delivered into the oocyte upon fertilization, giving credence to the notion that they may indeed play

a role in development (Ostermeier *et al.* 2004); however, the functional relevance of these molecules to fertilization and embryonic development has not yet been determined (Krawetz 2005). Testing the need for these sperm components in SCNT models will provide information about their function, as well as potentially improving cloning efficiency.

Expression of donor-cell-specific genes was previously observed in cloned mice, suggesting that nuclear reprogramming may be incomplete after nuclear transfer (Gao *et al.* 2003). This epigenetic memory can have adverse consequences for embryonic development, as demonstrated by mouse cloning experiments in which nuclear transfer embryos developed better in donor cell culture medium than in embryo culture medium (Gao *et al.* 2003). We observed that a somatic-specific gene (*TRYP8*) was inactivated more efficiently in PLCZ-activated embryos than in the chemically-activated embryos. Our finding that a more physiological activation stimulus (PLCZ) resulted in a reduction of somatic gene expression abnormalities provides evidence that oocyte activation may play a role in reprogramming the embryonic genome, leading to erasure of somatic cell epigenetic memory. Similarly, it was shown that a more-physiological pattern of $[Ca^{2+}]_i$ oscillations affected gene expression at the blastocyst stage (Ozil *et al.* 2006) and that an appropriate level of Ca^{2+} signal was required to induce translation of maternally stored mRNA in the early zygote (Ducibella *et al.* 2002), potentially affecting the reprogramming of the zygotic chromatin.

Trimethylation of histone H3 at lysine 27 is catalyzed by polycomb group complexes and is associated with stable and heritable gene silencing (Schuettengruber *et al.* 2007). In the present study, we found that levels of H3K27me3 in SCNT embryos were similar to IVF embryos when they were activated by a sperm-like stimulus (PLCZ) but higher when chemical activation methods were used. This observation agreed with our gene expression data, where chemically activated embryos showed higher levels of somatic gene expression, suggesting that embryos activated by chemical means may retain a somatic-like pattern of epigenetic arrangement compared with PLCZ-activated and fertilized embryos. The mechanism by which the activation system influenced the reprogramming of H3K27me3 represents an interesting area for future research. Moreover, given the importance of H3K27me3 in conferring stem cell identity to embryonic stem cells (Azuara *et al.* 2006, Boyer *et al.* 2006, Lee *et al.* 2006), it is tempting to speculate that aberrant H3K27me3, in cloned embryos activated by chemical means, may lead to abnormal cell differentiation, thus resulting in developmental abnormalities and embryonic lethality.

In summary, we have utilized an oocyte activation treatment (PLCZ cRNA injection) that closely recapitulated the calcium oscillation patterns observed after

fertilization. This treatment supported activation and *in vitro* development of bovine SCNT embryos at rates comparable with those induced by chemical activation and by fertilization. Also, embryos activated using this protocol had similar characteristics – in terms of cell number, ploidy, apoptosis, and chromatin modifications – to *in vitro* fertilized embryos. Moreover, our experiments provide evidence that the oocyte activation system chosen can, at the eight-cell and blastocyst stages, affect gene expression as well as chromatin modifications, implying that the activation stimulus has a role in nuclear reprogramming. Conversely, *U2AF1L1* expression was not detected in any of the SCNT groups, indicating that some abnormalities common to SCNT embryos persisted in clones activated by PLCZ cRNA injection. Allowing SCNT embryos activated with PLCZ to develop to further stages, and even to term, will likely provide more conclusive evidence on the involvement of the activation stimulus during nuclear reprogramming.

Materials and Methods

All chemicals were purchased from Sigma unless stated otherwise.

PLCZ cRNA preparation

A pBluescript vector containing the full-length coding sequence of murine PLCZ (mPLCZ) was linearized with EcoRI and used as a template for *in vitro* transcription by the T7 mMESSAGE mMACHINE High Yield Capped RNA Transcription Kit (Ambion, Austin, TX, USA), following the manufacturer's instructions. A poly(A) tail was then added to the cRNA using a Poly(A) Tailing Kit (Ambion). The cRNA was purified using the MEGAclear Kit (Ambion) and stored at -80°C in single-use aliquots. Just before use, the cRNA was thawed on ice, heated to 85°C for 3 min, and centrifuged at $16\,000\text{ g}$ at 4°C for 5 min.

Somatic cell nuclear transfer

Oocytes were obtained from abattoir-derived ovaries and matured *in vitro* as previously described (Ross *et al.* 2006). SCNT was performed as described (Ross *et al.* 2006). Briefly, cumulus cells were removed by vortex agitation in media containing 1 mg/ml hyaluronidase 16–18 h after oocyte maturation. Oocyte enucleation was performed by aspirating the metaphase II chromosomes in a small volume of surrounding cytoplasm. Donor cells were dissociated by treatment with 10 IU/ml of pronase in HECM-Hepes (HH) media (Seshagiri & Bavister 1989) for 5 min. A single cell was inserted into the perivitelline space of the enucleated oocyte and fused in calcium-free sorbitol fusion medium by applying a single direct current pulse of 234 volts/mm for 22 μs .

Activation and embryo culture

Activation of fused nuclear transfer units (NTUs) was performed 2 h after fusion. Three different activation protocols were implemented: 1) ionomycin/DMAP, 2) ionomycin/CHX, and 3) PLCZ. In groups 1 and 2, NTUs were treated with 5 μ M ionomycin (Calbiochem, San Diego, CA, USA) for 4 min, followed by incubation in potassium simplex optimized medium (KSOM) medium containing either 10 μ g/ml CHX and 5 μ g/ml CB for 5 h (ionomycin/CHX), or 2 mM DMAP for 4 h (ionomycin/DMAP). Activation using PLCZ was performed by intracytoplasmic injection of ~6 to 8 pl of 1 μ g/ μ l mPLCZ cRNA, as previously described (Ross *et al.* 2008b). Then, to prevent the extrusion of the second polar body, NTUs were cultured for 5 h in KSOM containing 7.5 μ g/ μ l CB. After activation, the NTU were rinsed several times in HH medium and cultured in 400 μ l drops of KSOM medium supplemented with 3 mg/ml of BSA under mineral oil at 38.5 °C and 5% CO₂ in air for 7 days. On day 3 (NT=day 0), culture medium was supplemented with 10% v/v fetal bovine serum (FBS). Eight-cell and blastocyst-stage embryos were collected 50 and 180 h after activation respectively.

Fertilized control embryos were produced by IVF using Tyrode's albumin lactate pyruvate-based medium (Parrish *et al.* 1986) and cultured under the same conditions as the SCNT embryos.

Intracellular calcium monitoring

Intracellular Ca²⁺ concentrations were measured using Fura Red. After PLCZ injection, the zygotes were loaded in HH medium containing 2 μ M Fura Red AM (Invitrogen), 0.02% v/v Pluronic F-127 (Invitrogen) and 0.5 M sulfapyrazone for 10 min at 38.5 °C. After loading, oocytes were placed in 50 μ l drops of protein-free HH medium containing 0.5 M sulfapyrazone on a Petri dish with a glass bottom and covered with mineral oil. During the first 5 h after PLCZ injection, the medium was also supplemented with 7.5 μ g/ml CB. The Petri dish was placed on a heated stage on a Nikon TE2000-U microscope (Nikon, Tokyo, Japan). A 120W metal halide lamp (X-Cite 120, EXFO, Quebec, Canada) provided the excitation light through fiber optics, and excitation wavelengths were 440 and 490 nm. Wavelengths greater than 600 nm were collected through a 20X objective by an EMCCD camera fitted with on-chip multiplication gain (Cascade 512B, Roper Scientific, Tucson, AZ, USA). Fluorescence intensity ratios (440/490 nm) were measured every 20 s using Metamorph software (Universal Imaging Corp., Downingtown, PA, USA).

Blastocyst differential staining and TUNEL assay

The zona pellucida of each blastocyst was removed by incubation in 10 IU/ml pronase for 2 min. After thoroughly rinsing the embryos in HH medium, they were exposed for 10 s to 0.2% v/v Triton X-100 in PBS, (Gibco) containing 2 mg/ml BSA. The embryos were then incubated for 15 min in PBS-BSA containing 10 μ g/ml bisbenzimidazole and 30 μ g/ml propidium iodide. After staining, the embryos were fixed in 4% w/v paraformaldehyde for 15 min and stored at 4 °C, for no more

than 7 days, until a TUNEL assay was performed. TUNEL assays were by the In Situ Cell Death Detection Kit (Roche Applied Science) following manufacturer instructions. Briefly, the embryos were exposed to the labeling solution containing the terminal deoxynucleotidyl transferase and fluorescein-labeled nucleotide mixture for 1 h at 37 °C in a humid chamber. The embryos were then treated with RNase A (50 IU/ml) for 30 min at 37 °C. Embryos treated with RQ1 DNase (10 IU/ml) were used as a positive control, and negative controls were incubated in labeling solution omitting the enzyme. After intensive washing in PBS-BSA, the embryos were mounted in a small drop of ProLong Gold antifade solution (Invitrogen) and evaluated under epifluorescence microscopy. TE cells were observed as red nuclei; ICM cells, as blue nuclei; and TUNEL-positive cells, as green nuclei.

Blastocyst chromosomal analysis

Embryos were incubated for 12–14 h in KSOM-BSA plus 5% v/v FBS containing 0.05 μ g/ml demecolcine. Then, embryos were exposed to a hypotonic 0.075 M KCl solution for 5 min to induce nuclear swelling. Subsequently, embryos were placed on a clean glass slide in a small volume of media. A methanol-acetic acid solution (1:1) was dropped on top of embryos while gently blowing with the slides placed under the stereoscope. Just before the solution dried, the slide was submerged in a 3:1 methanol-acetic acid solution for 1 h and then allowed to dry at room temperature for 24 h. After drying, samples were mounted using Prolong Gold antifade solution with 4',6-diamidino-2-phenylindole (Invitrogen). Chromosome spreads were evaluated under epifluorescence at 1000X magnification with oil immersion optics (Nikon). Embryos were classified as being haploid, diploid, triploid, tetraploid, polyploid, and mixoploid.

Cell number determination of live embryos

The total number of cells in the blastocysts used for gene expression analysis was determined by live confocal microscopy, as previously described (Ross *et al.* 2006). Briefly, the nuclei were stained by incubation in HH medium containing 5 μ M SYTO 16 (Molecular Probes, Eugene, OR, USA) for 15 min. Then, the embryos were placed with the ICM facing the objective lens between two coverslips separated from each other by 150 μ m and imaged using a spinning-disk confocal system (CARV, Atto Bioscience Inc., Rockville, MD, USA) mounted on a Nikon TE2000-U microscope. A Z-stack of the embryo was acquired every 5 μ m, and the images were processed for three-dimensional deconvolution using Autoquant (Media Cybernetics, Inc., Silver Spring, MD, USA) and analyzed using Metamorph software (Universal Imaging Corp.). All nuclei were marked by drawing a contour on the image for each focal plane and counted.

Quantitative RT-PCR

Groups of five eight-cell embryos and individual blastocysts were lysed in 20 μ l of extraction buffer, and then incubated at 42 °C for 30 min followed by centrifugation at 3000 g for 2 min

and stored at -80°C . Before RNA extraction, each sample was spiked with $2\text{ }\mu\text{l}$ of $250\text{ fg}/\mu\text{l}$ HcRed1 cRNA, used as an exogenous control (Bettegowda *et al.* 2006), and $50\text{ }\mu\text{g}$ tRNA as a carrier. Total RNA was extracted from each sample using the PicoPure RNA Isolation Kit (Arcturus, Carlsbad, CA, USA) according to the manufacturer's instructions. Residual genomic DNA was removed by DNase I digestion using an RNase-Free DNase Set (Qiagen). RNA was eluted from the purification column using $11\text{ }\mu\text{l}$ nuclease-free water (Ambion). RNA was then primed with oligo-dT (Invitrogen) and converted into cDNA using Superscript II (Invitrogen) following the manufacturer's instructions. Each RT reaction was finally diluted with nuclease-free water to a final volume of $60\text{ }\mu\text{l}$.

The quantification of all gene transcripts was done by real-time quantitative RT-PCR using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Absolute quantification using this method is described elsewhere (Li & Wang 2000, Whelan *et al.* 2003). Primer sequences for all the genes are shown in Supplementary Table 1, which can be viewed online at www.reproduction-online.org/supplemental/.

Each reaction mixture consisted of $2\text{ }\mu\text{l}$ cDNA, $5\text{ }\mu\text{mol}$ each of forward and reverse primers, $7.5\text{ }\mu\text{l}$ of nuclease-free water, and $12.5\text{ }\mu\text{l}$ SYBR Green PCR Master Mix in a total reaction volume of $25\text{ }\mu\text{l}$. Reactions were performed in duplicate for each sample in an ABI Prism 7000 Sequence Detection System (Applied Biosystems). Dissociation curves were performed after each PCR run to ensure that a single PCR product had been amplified.

The copy number of HcRed1 cRNA was determined for each sample using a standard curve constructed from the plasmid pHc-Red1-Nuc (Clontech). For HcRed, *GAPDH*, *POU5F1*, *NANOG*, *SOX2*, *CDX2*, and *FGFR2*, plasmids containing the partial cDNAs were used to construct standard curves using tenfold serial dilutions. For *TRYF8*, *GLUT1*, *DSC2*, and *U2AF1L2*, a relative standard curve was used to determine abundance in arbitrary units using serial dilutions of amplified cDNA from a pool of bovine IVF and SCNT blastocysts and fibroblasts.

For each measurement, threshold lines were adjusted to intersect amplification lines in the exponential portion of the amplification curve using the automatic setting of the thermocycler program. HcRed1 (the external control) abundance was determined in each sample and used to normalize for differences in RNA extraction and RT efficiency. Blastocyst embryo samples were further normalized to the total cell number of each individual embryo.

Immunostaining of embryos

Embryos were washed in PBS containing $1\text{ mg}/\text{ml}$ of polyvinyl alcohol (PVA), fixed with 4% w/v paraformaldehyde for 15 min in PBS (Gibco) and stored at 4°C in PBS containing $1\text{ mg}/\text{ml}$ of PVA for no longer than 3 weeks. Embryos were permeabilized in 1% v/v Triton X-100 for 30 min at room temperature, then incubated with Image-iT FX signal enhancer (Invitrogen) for 30 min , and blocked with 10% v/v normal goat serum for 2 h . Embryos were incubated overnight at 4°C in 1% w/v BSA and primary antibodies against H3K27me3; (ab6002; Abcam, Cambridge, UK) and acetylated lysine 5 of histone H4 (H4K5Ac; 07-327; Upstate). After 6 h washing in PBS

containing 0.1% v/v Triton X-100, embryos were incubated with secondary antibodies conjugated with Alexa 488 and Alexa 594 (Invitrogen) for 1 h at room temperature. DNA was visualized by bisbenzimid staining. For imaging, embryos were mounted in $11\text{ }\mu\text{l}$ antifade solution and compressed with a coverslip. Imaging was performed by a spinning-disk confocal system mounted on a Nikon TE-2000 microscope at $40\times$ (numerical aperture (NA) 1.3) and $100\times$ (NA 1.3) magnifications. Optical sections every $1\text{ }\mu\text{m}$ were acquired for each embryo. Metamorph software was used for image acquisition and analysis. Fluorescence intensity determinations were performed as previously described (Ross *et al.* 2008a). All sections were combined by a maximum projection, and each nucleus was delineated under the blue channel (nuclear staining). Also, two different cytoplasmic areas were delineated to use as background fluorescence. The regions were then transferred to the red and green channels, and the average pixel intensity was calculated by the software for each region. For analysis, each region's fluorescence intensity was divided by the average of the two cytoplasmic regions.

Statistical analysis

Continuous response variables were analyzed by ANOVA using the MIXED procedure of SAS (Cary, NC, USA). The models included treatment as fixed effect and a random effect of manipulation day. Rates of embryonic development to cleavage and blastocyst stage were evaluated using a generalized linear model methodology, including the fixed effect of treatment and the random effect of replicate. The analyses were implemented using the GLIMMIX procedure of SAS, assuming a binomial distribution of the response variables and a logit link function.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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