

Development, embryonic genome activity and mitochondrial characteristics of bovine–pig inter-family nuclear transfer embryos

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Abstract

The best results of inter-species somatic cell nuclear transfer (iSCNT) in mammals were obtained using closely related species that can hybridise naturally. However, in the last years, many reports describing blastocyst development following iSCNT between species with distant taxonomical relations (inter-classes, inter-order and inter-family) have been published. This indicates that embryonic genome activation (EGA) in xeno-cytoplasm is possible, albeit very rarely. Using a bovine–pig (inter-family) iSCNT model, we studied the basic characteristics of EGA: expression and activity of RNA polymerase II (RNA Pol II), formation of nucleoli (as an indicator of RNA polymerase I (RNA Pol I) activity), expression of the key pluripotency gene *NANOG* and alteration of mitochondrial mass. In control embryos (obtained by IVF or iSCNT), EGA was characterised by RNA Pol II accumulation and massive production of poly-adenylated transcripts (detected with oligo dT probes) in blastomere nuclei, and formation of nucleoli as a result of RNA Pol I activity. Conversely, iSCNT embryos were characterised by the absence of accumulation and low activity of RNA Pol II and inability to form active mature nucleoli. Moreover, in iSCNT embryos, *NANOG* was not expressed, and mitochondria mass was significantly lower than in intra-species embryos. Finally, the complete developmental block at the 16–25-cell stage for pig–bovine iSCNT embryos and at the four-cell stage for bovine–pig iSCNT embryos strongly suggests that EGA is not taking place in iSCNT embryos. Thus, our experiments clearly demonstrate poor nucleus–cytoplasm compatibility between these animal species.

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Introduction

During early embryogenesis, a precise and accurate communication between nucleus and cytoplasm determines the success of such critical step of embryo development as embryonic genome activation (EGA). In inter-species somatic cell nuclear transfer (iSCNT), the situation is even more complicated as the donor nucleus and recipient cytoplasm originate from different species. Thus, during the maternal-to-embryonic transition (MET), activation of embryonic nuclei, which bear the genetic information of one species, critically depends on the stock of proteins and transcripts in the recipient cytoplasm (Schultz 1993, Minami *et al.* 2007), which is derived from another species. Initiation of gene transcription involves many factors, and the absence or the species incompatibility of just one of them can block the subsequent transcription (Heix *et al.* 1997).

Nevertheless, despite the possibility of nucleus–cytoplasm incompatibility, iSCNT represents a plausible experimental scheme especially when oocytes are extremely scarce. In 1999, the work by Dominko *et al.* (1999) raised hopes about the possibility of using oocytes from different species as recipients of somatic cell nuclei to save endangered species or to create embryonic stem cells for human cell therapy. Species that hybridise naturally are more likely to perform well in iSCNT experiments. This is understandable, since the natural production of living hybrid offspring shows that a certain nuclear–cytoplasmic compatibility exists between the two species (Mastromonaco *et al.* 2007). As a rule, iSCNT in mammals is more efficient when donor and recipient cells are from closely related species. For instance, inter-subspecies SCNT produced healthy offspring of Boer goat (Jian-Quan *et al.* 2007) and grey wolf (Kim *et al.* 2007). iSCNT embryos derived

from mouflon (*Ovis orientalis musimon*), nucleus donor cells and sheep (*Ovis aries*) oocytes (Loi *et al.* 2001) could also develop to term. Wildcat (*Felis silvestris lybica*; Gomez *et al.* 2004) and sand cat (*Felis margarita*; Gomez *et al.* 2008) were produced using domestic cat (*Felis catus*) oocytes. iSCNT embryos derived from gaur (*Bos gaurus*) adult nucleus donor cells and bovine (*Bos taurus*) oocytes implanted and fetuses developed up to 200 days (Lanza *et al.* 2000). Inter-genus SCNT embryos derived from leopard cat (*Prionailurus bengalensis*) nucleus donor cells, and domestic cat oocytes were able to implant and form fetuses (Yin *et al.* 2006).

On the other hand, many studies have reported production of iSCNT morulae and blastocysts (BL) also when nucleus donor cells and recipient oocytes had a very distant taxonomical relation, like in the case of inter-family bovine–pig (Dominko *et al.* 1999, Uhm *et al.* 2007), inter-order cat- and panda–rabbit (Wen *et al.* 2005), camel- and Tibetan antelope–rabbit (Zhao *et al.* 2006), human–rabbit (Shi *et al.* 2008), dog–pig (Sugimura *et al.* 2009), tiger–pig (Hashem *et al.* 2007), human–bovine (Chang *et al.* 2003, Illmensee *et al.* 2006, Li *et al.* 2008) or inter-classes chicken–rabbit (Liu *et al.* 2004) combinations. However, this approach remains ineffective.

The aim of the present study was to investigate pre-implantation development in bovine–pig iSCNT embryos that have been previously shown to develop to blastocyst stage at the rate of 4% (Uhm *et al.* 2007) to 8% (Dominko *et al.* 1999). Indeed, very few data are available on embryonic gene function before and at the time of EGA in iSCNT embryos (Arat *et al.* 2003, Bjerregaard *et al.* 2007, Chung *et al.* 2009, Hamilton *et al.* 2004, Kim *et al.* 2004, Li *et al.* 2008, Wang *et al.* 2009), and all show abnormal genome activation and gene expression profiles in the iSCNT embryos studied. It is likely that the ineffectiveness of the iSCNT approach may be linked to abnormal (or absence of) EGA in such embryos due to aberrant nucleus–cytoplasm cross-talk. Since *Bos taurus* and *Sus scrofa domestica* have a rather distant (inter-family) taxonomical relation and EGA occurs at different cell stages (8–16-cell stage in bovine and four-cell stage in porcine embryos), the pig–bovine iSCNT model may thus represent a useful model for investigating the nucleus–cytoplasm interaction during early embryo development. We have focused especially on nucleus remodelling, embryo development, donor cell-specific gene (collagen type VI α 1 gene (*COL6A1*)) fate and EGA during MET, with special attention to RNA polymerase II (RNA Pol II) activity, nucleoli formation (as an indicator of RNA polymerase I (RNA Pol I) activity) and activation of the pluripotency gene *NANOG*. Finally, we have investigated the behaviour of mitochondria under xeno-nuclear control.

Results

Nucleus remodelling in bovine and porcine ooplasm

To investigate pre-implantation development, we created both intra-species (bovine or porcine NT) and inter-species (bovine–pig or pig–bovine iSCNT) embryos by NT. Remodelling is believed to be an essential event in reprogramming of the genetic material after NT. This phenomenon involves structural changes in the donor nucleus, such as nuclear envelope breakdown, premature chromosome condensation (PCC) as well as pronuclear development and swelling (Tsunoda *et al.* 1989). The analysis of NT and iSCNT embryos (Supplementary Table 1, see section on supplementary data given at the end of this article) showed that about 90–100% of fused embryos, irrespectively of the origin of the recipient cytoplasm and of the nucleus donor cell, exhibited PCC 1 h post-fusion and swollen nuclei with a pronucleus-like appearance 3 h after activation. These results indicate that remodelling of the donor cell nucleus in the recipient cytoplasm is highly efficient and not species specific.

In vitro development of iSCNT embryos

In the first set of experiments, we investigated the developmental potential of iSCNT embryos reconstructed from porcine donor cells and bovine recipient oocytes. As a control, intra-species porcine NT embryos were used. We tested two porcine adult cell lines (PAF1 and PAF2) and one foetal fibroblast line (PFF) as nucleus donors for both NT and iSCNT embryos. In the control group, the proportion of embryos which had developed to the blastocyst stage was 20–38% at day 6 post-activation (Supplementary Table 2, see section on supplementary data given at the end of this article). Conversely, iSCNT embryos did not develop beyond the 16–25-cell stage (72 h after activation), and eventually, development was blocked. There was no sign of compaction or blastulation during further culture till day 8. No difference between SOFaa and CR1aa medium in the ability to support the development of iSCNT embryos was found.

We then repeated the same experiments using bovine foetal (BFF) as well as adult male (BAFm) and female (BAFf) fibroblasts as nucleus donors and porcine oocytes as recipients. The proportion of control bovine NT embryos that developed to blastocyst stage was about 50% at day 7 indicating a high efficiency of bovine NT and the suitability of the donor cell lines used (Supplementary Table 3, see section on supplementary data given at the end of this article). At 72 h after activation, all advanced embryos were at the four-cell stage in both NT and iSCNT groups. However, at 96 h, almost half (47%, $n=38$) of control NT embryos possessed eight or more cells while all advanced iSCNT embryos (50–67.9%) were blocked at the 4–6-cell

stage (4.1 ± 0.2 , $n=30$; 4–6 nuclei) indicating developmental arrest. There was no sign of compaction or blastulation during further culture till day 7.

Next, we counted the number of cells and nuclei in each pig–bovine iSCNT embryo because they just passed the porcine EGA stage. This step was necessary also as high level of cellular abnormality can be expected in iSCNT embryos and blastomere fragmentation can occur (Lorthongpanich *et al.* 2008). We analysed the most advanced embryos at 48 h (i.e. embryos made up of ≥ 4 cells), 72 h (≥ 8 cells) and 96 h (≥ 8 cells) after activation. There was no difference in the number of cells (10.2 ± 2.1) and nuclei (9.6 ± 2.3) between bovine NT and pig–bovine iSCNT embryos at 48 h. At 72 h, the average number of nuclei in NT embryos (Table 1) was equal to cell number and was significantly lower than in pig–bovine iSCNT embryos. At the same time, the average number of nuclei in pig–bovine iSCNT embryos (Table 1) was significantly higher than the number of blastomeres because many binucleate blastomeres were observed (Supplementary Figure 1, see section on supplementary data given at the end of this article). Binucleate blastomeres were rarely seen in the control group (Table 1), whereas the average number of anucleate blastomeres, which is a marker of embryo fragmentation, was equal in NT and iSCNT embryos. DAPI staining of paraformaldehyde-fixed embryos also revealed a tremendous difference in nuclear morphology: in NT embryos, nuclei were stained homogeneously, whereas, in iSCNT embryos, many nuclei with condensed chromosomes were detected (Fig. 1). Since chromosome condensation can also occur during apoptosis and one of the earliest events in apoptosis is the translocation of phosphatidylserine to the outer membrane, we stained fixed embryos with Annexin V, which binds to phosphatidylserine, to detect apoptotic cells in embryos at 96 h post-activation. However, there was no difference in the apoptotic rate of NT and iSCNT embryos (data not shown). At 96 h,

the number of nuclei in bovine NT embryos increased significantly, while it did not change in pig–bovine iSCNT embryos. This clearly indicates that development in iSCNT embryos was blocked.

Altogether these results suggest that in iSCNT embryos, early cleavage is governed by the cytoplasm of the recipient oocyte and by maternally inherited factors that engage in cell division. Porcine oocytes controlled the development of bovine–pig iSCNT embryos arresting them at the time of porcine MET (i.e. four-cell stage), whereas bovine oocytes blocked the development of pig–bovine iSCNT embryos at the time of bovine MET during the fourth cell cycle (8–16-cell stage).

Cytoplasm controls the expression of fibroblast-specific COL6A1

We then asked how nuclear genes with tissue-specific inducible promoters that should be active in the somatic donor cells but inactive in the oocyte recipients (Memili & First 1998) behaved in iSCNT embryos. To this aim, we evaluated the expression of the fibroblast-specific COL6A1 (Supplementary Figure 5, see section on supplementary data given at the end of this article) in bovine NT and in pig–bovine iSCNT embryos starting from the two-cell stage. COL6A1 is highly expressed in bovine and porcine adult fibroblasts, and can be detected by RT-PCR from the equivalent of one fibroblast. COL6A1 mRNA was not detected in any of the embryos analysed, indicating that the bovine cytoplasm not only blocked *de novo* transcription of a fibroblast-specific gene irrespectively of the species affiliation of the nuclear donor but also effectively degraded transcripts inherited with the fibroblast cytoplasm. These preliminary experiments seem to confirm the data by Li *et al.* (2007) on the silencing of the fibroblast-specific vimentin and collagen genes in early yak–bovine iSCNT embryos.

Table 1 Number of cells and nuclei in bovine nuclear transfer (NT) and pig–bovine inter-species somatic cell nuclear transfer (iSCNT) embryos. Mean \pm s.d.

Parameters	Time of <i>in vitro</i> culture			
	72 h		96 h	
	NT	iSCNT	NT	iSCNT
Number of embryos	46	62	45	32
Number of cells/embryo	8–17	8–25		
Number of cells/embryo, M \pm s.d.	11.2 ± 2.7^a	13.8 ± 3.9^{bA}		
Number of nuclei/embryo, M \pm s.d.	11.2 ± 2.6^a	15 ± 4.4^{bB}	19.7 ± 7.2^c	15.2 ± 3.8^b
% of embryos with:				
Anucleate cells	22.6	23.9		
Binucleate cells	10.3 ^a	47.3 ^b		
≥ 16 cells	4.3 ^a	12.9 ^{a*}		
≥ 16 nuclei	2.2 ^a	30.6 ^b		

Values with different letters in one row (a, b, c) are significantly different by *t*-test, $P < 0.01$. Values with 'a' versus 'a*' in one row describe a tendency to be different, $P = 0.12$. Values with different letters in one column (A, B) are significantly different, $P < 0.01$.

Assessment of EGA

RNA Pol II

Since the previous experiments indicate that iSCNT embryos' development is blocked at the time of EGA, we decided to analyse the events associated with the activation of transcription. In *Xenopus laevis*, EGA initiates with the activation of class II genes (mRNA), followed by class III genes (tRNA) and finally class I genes (rRNA; Shiokawa *et al.* 1989, 1994). In eukaryotes, RNA Pol II is responsible for transcription of mRNAs and of most of the small nuclear RNAs. Thus, we used an antibody that recognises RNA Pol II irrespectively of its phosphorylation status to visualise its accumulation, and RNA-FISH to estimate RNA Pol II activity through the detection of poly-adenylated (poly) mRNA accumulation in the nuclei. Moreover, since some aberrations can be found in genes' expression of intra-species NT embryos, IVF embryos were included in this set of experiments as an additional control group.

We studied the most advanced IVF, NT and iSCNT embryos at 24, 48, 72 and 96 h after activation. In the nuclei of IVF and NT embryos, RNA Pol II (Fig. 2; Supplementary Figure 2, see section on supplementary data given at the end of this article) and poly(A) RNAs (Fig. 3; Supplementary Figure 3, see section on supplementary data given at the end of this article) were found at all studied stages of early embryo development and labelling intensity clearly increased at 72 and 96 h post fertilisation/activation in comparison with the earlier time points. Conversely, iSCNT embryos were characterised by low level of RNA Pol II expression and low transcriptional activity in the nuclei starting from the two-cell stage, and both RNA Pol II expression and activity did not increase at the time of EGA (Figs 2 and 3; Supplementary Figures 2 and 3).

Activation of gene expression in iSCNT embryos

Next, we assessed whether functional transcripts could be generated in iSCNT embryos, despite the low level of RNA Pol II activity by using porcine green fluorescent protein (GFP)-positive foetal fibroblasts (GFP+ cells under the control of the constitutive *PGK* promoter) as nucleus donor cells for pig–bovine iSCNT embryos. Accumulation of GFP was detected in iSCNT embryos starting from the one-cell stage (24 h after activation), and more than 80% of pig–bovine iSCNT embryos (Supplementary Table 2) showed accumulation of GFP at day 7 (Supplementary Figure 4, see section on supplementary data given at the end of this article). This indicates that low level of RNA Pol II transcription was present in iSCNT embryos.

Another event associated with RNA Pol II activity but crucially dependent on EGA is the expression of *NANOG*, a key pluripotency gene (Brevini *et al.* 2006, Gandolfi *et al.* 2006). We were unable to detect *NANOG* mRNA in pig–bovine (Supplementary Figure 5D) iSCNT embryos at any of the developmental stages (2-, 4-, 8- and 16-cell) analysed. These data confirm that no or highly aberrant genome activation takes place at the porcine MET (4–8-cell) or at the bovine MET (8–16 cell).

RNA Pol I activation and nucleolar maturation

In mammals, the fact that transcriptional activation of class I genes (rRNA) depends on the previous activation of class II genes (mRNA; Shiokawa *et al.* 1989, 1994) was recently confirmed in bovine (Maddox-Hyttel *et al.* 2007, Svarcova *et al.* 2007) and porcine embryos (Maddox-Hyttel *et al.* 2007). Specifically, RNA Pol I as well as other key nucleolar proteins (Svarcova *et al.* 2007) are *de novo* expressed from embryonic genome, and consequently, rRNA gene activation and the associated nucleolus formation have been suggested as

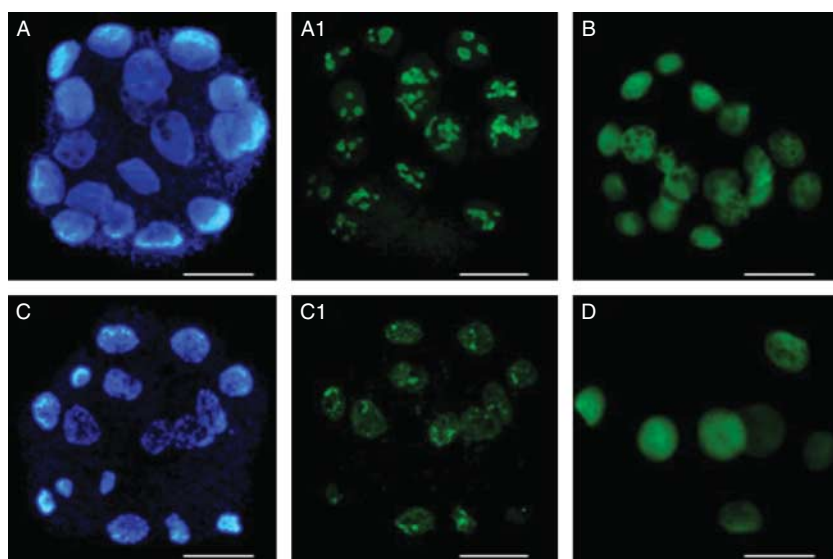


Figure 1 Bovine NT (A, A1, B and D) and pig–bovine iSCNT embryos (C and C1) labelled with anti-nucleolin (C23) antibody (A1, B, C1 and D). Bovine NT embryo (A1) and pig–bovine iSCNT embryo (C1) were counterstained with DAPI (A and C) at 96 h after activation. DAPI highlighted the presence of (A) homogeneously stained nuclei in NT embryo and (C) chromatin condensation in the nuclei of iSCNT embryo. C23 labelling revealed (A1) polygonal mature nucleoli in the nuclei of bovine NT embryo, which (B) disappeared following treatment with 0.2 μg/ml actinomycin D (AD) for 3 h; (D) the lack of nucleolar formation in bovine NT embryo after 48 h treatment with 2 μg/ml AD; (C1) the absence of mature nucleoli in pig–bovine iSCNT embryo. Scale bars, 100 μm.

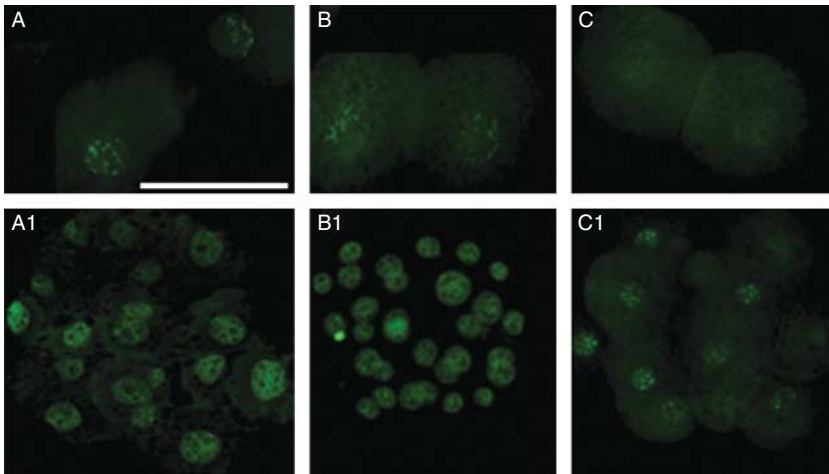


Figure 2 RNA Pol II was visualised with the CTD4H8 antibody that detects both phosphorylated and unphosphorylated RNA Pol II in bovine IVF (A and A1), NT (B and B1) and pig-bovine iSCNT (C and C1) embryos. The embryos were fixed at the two-cell stage (A–C) and at the most advanced stages at 96 h after insemination/activation (A1, B1 and C1). CTD4H8 labelling revealed the presence of RNA Pol II in the nuclei of (A) IVF, (B) NT and (C) iSCNT two-cell stage embryos, significant accumulation of RNA Pol II in the nuclei of (A1) IVF and (B1) NT embryos, and (C1) the lack of RNA Pol II accumulation in iSCNT embryos. Scale bar, 100 μ m.

markers of EGA in mammalian embryos (Bjerregaard *et al.* 2007). Nucleolin is a multifunctional maternally inherited nucleolar protein (Svarcova *et al.* 2007) engaged in the late processing of rRNA that colocalises with RNA Pol I to nucleoli. We thus used an antibody against nucleolin (C23) to evaluate nucleolus formation in the more advanced NT and iSCNT embryos at 96 h after activation (Figs 1 and 4). NT embryos were characterised by the presence of intensely labelled polygonal and shell-like mature nucleoli. Conversely, pig-bovine (Fig. 1C1) and bovine-pig (Fig. 4D) iSCNT embryos were characterised by prevailing nucleoplasmic

localisation of nucleolin with occasional concentration in faintly stained half-rings or rings in bovine nuclei or in several dots or rings in some porcine nuclei.

This result, together with the data on RNA Pol II and *NANOG*, suggests that the embryonic genome is not properly activated in iSCNT embryos. To further test this hypothesis, we incubated bovine NT embryos with actinomycin D (AD) and porcine NT embryos with α -amanitin (AA). Long-term (48 h) incubation with AD or AA effectively blocked EGA in NT embryos as indicated by the absence of mature nucleoli (Figs 1D and 4C) and the appearance of nuclei with condensing chromatin in

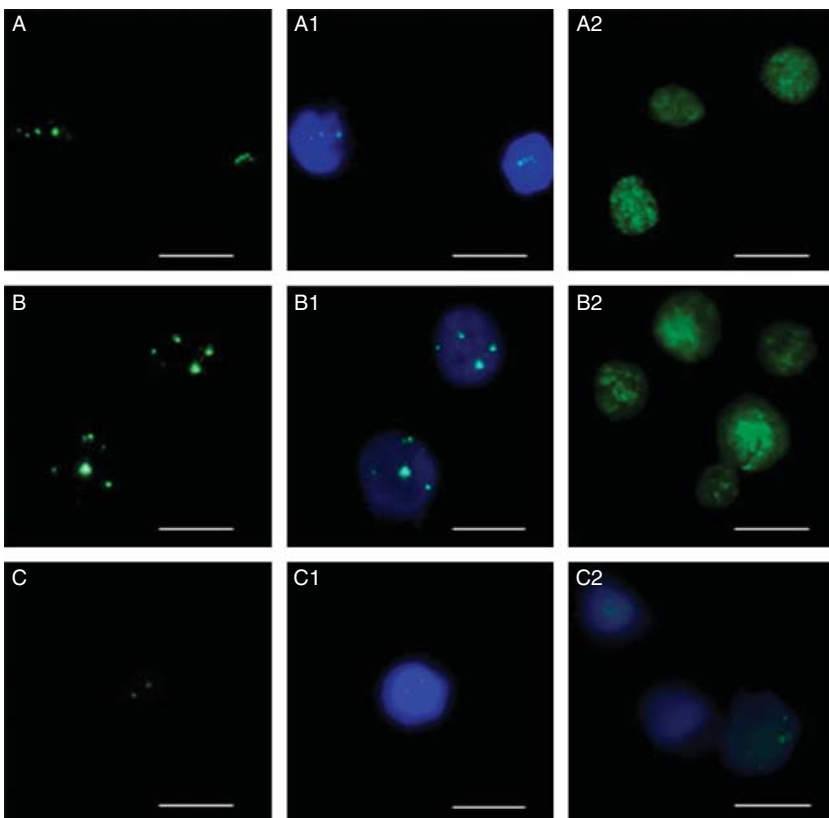


Figure 3 RNA Pol II activity, as determined by the accumulation of polyadenylated transcripts in the nuclei, was assessed by RNA FISH with a fluorescein-labelled oligo dT probe in bovine IVF (A, A1 and A2), NT (B, B1 and B2) and pig-bovine iSCNT (C, C1 and C2) embryos. Embryos were fixed and labelled at the two-cell stage (A–C) and at the most advanced stages at 96 h after insemination/activation (A2, B2 and C2). Nuclei were counterstained with DAPI for DNA visualisation. Dot-like labelling of polyadenylated transcripts in the nuclei of two-cell stage (A) IVF, (B) NT and (C) iSCNT embryos and localisation of polyadenylated transcripts in DAPI-stained nuclei of (A1) IVF, (B1) NT and (C1) iSCNT embryos. Accumulation of polyadenylated transcripts in the nuclei of 8–16-cell stage (A2) IVF, (B2) NT embryos and (C2) dot-like labelling in the nuclei of iSCNT embryos. Scale bars, 100 μ m.

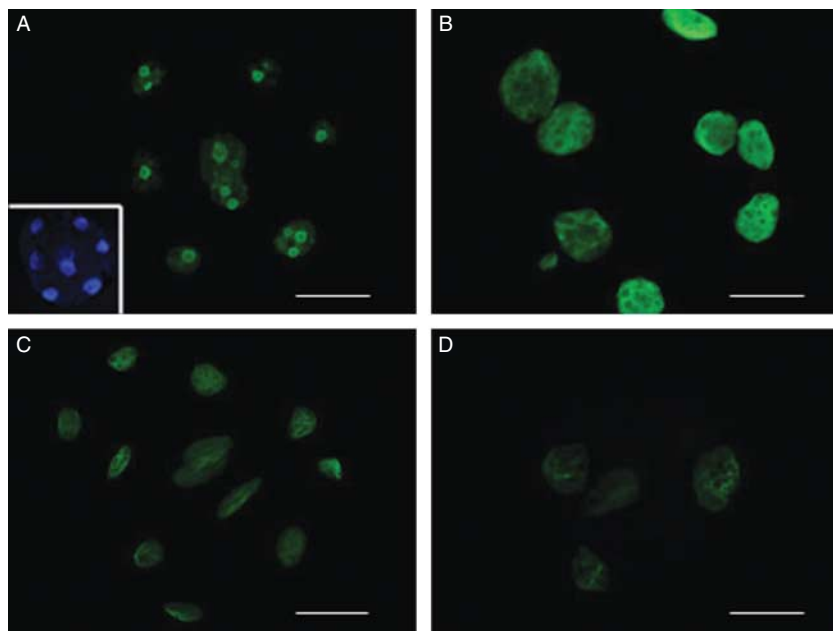


Figure 4 Porcine NT (A–C) and bovine–pig iSCNT (D) embryos labelled with anti-nucleolin (C23) antibody at 96 h after activation: (A) shell-like mature nucleoli in the nuclei of porcine NT embryo (in the corner the DAPI-staining of the same embryo), (B) disappearance of functional nucleoli from the nuclei of porcine NT embryo after 3 h treatment with 0.2 μ g/ml AD; (C) the lack of nucleolar formation in porcine NT embryo after 48 h treatment with α -amanitin; (D) the absence of mature nucleoli in bovine–pig iSCNT embryo. Scale bars, 100 μ m.

bovine NT embryos (not shown). Few faintly stained ring-like structures labelled with anti-nucleolin antibody could be found in some nuclei both in bovine and porcine NT embryos. Short incubation (3 h) with AD, to block RNA Pol I activity, resulted in complete disappearance of mature nucleoli (Figs 1B and 4B), nucleolin translocation into the nucleoplasm and few weakly anti-nucleolin-stained rings in some blastomeres. Thus, the formation of mature nucleoli in NT embryos depends on the synthesis of RNA Pol I during EGA and RNA Pol I-guided transcription of rRNA. The similar appearance of iSCNT embryos and AA- or AD-treated NT embryos suggests that in iSCNT embryos, the embryonic genome is not properly activated, RNA Pol I cannot be transcribed and as a result functional nucleoli cannot be formed.

Assessment of mitochondrial changes in NT and iSCNT embryos during development

Another important issue in iSCNT embryo development is the interaction of the nucleus with complex cytoplasmic organelles like mitochondria. Since mitochondria are involved in a broad range of critical functions (energy supply, cell signalling and apoptosis), inadequate or perturbed mitochondrial function may adversely affect SCNT efficiency (Hiendleder *et al.* 2005). Moreover, Lee *et al.* (2002) have shown that the increase in mitochondrial mass depends on cytoplasmic protein synthesis. Thus, changes in the mitochondrial mass in embryos are most probably linked to EGA.

To follow changes in mitochondria, we stained iSCNT and NT embryos with the cationic dye JC-1 at 48, 72 and 96 h after activation. JC-1 exists in a monomeric form, emitting at 527 nm (green fluorescence) after excitation

at 490 nm. In the cell, it corresponds to the low mitochondrial membrane potential (<100 mV) and characterises relative mitochondrial mass (Mancini *et al.* 1997). In the presence of high membrane potential (>140 mV), JC-1 forms so called J-aggregates that are associated with a large shift in emission (590 nm, red fluorescence). The ratio of red/green fluorescence characterises the inner mitochondrial membrane potential (Reers *et al.* 1991). We have found that at 48 h, NT and iSCNT embryos possessed equal relative mitochondrial mass (Fig. 5) and J-aggregate content (not shown). However, at 72 and 96 h, both parameters were significantly lower in iSCNT embryos than in their NT counterparts ($P < 0.05$). We then asked whether this discrepancy could be linked to the arrest of development and aberrant EGA observed in iSCNT embryos. To this aim, we inhibited genome transcription with AA in porcine and bovine NT embryos at 48 h for 24 h, and then stained them with JC-1 at 72 h. AA treatment led to significantly lower values of mitochondrial mass than in untreated NT embryos confirming that the most likely reason of low mitochondrial parameters in iSCNT embryos is the aberrant EGA.

Discussion

In the present manuscript, we have tried to characterise the main events of EGA, namely activation of RNA Pol II transcription at the time of MET that should lead to subsequent transcription of RNA Pol I and pluripotency genes. Any serious aberrations of these events cause the developmental failure of the embryos. Our results indicate that the most likely cause of pig–bovine and bovine–pig iSCNT embryos developmental failure is the

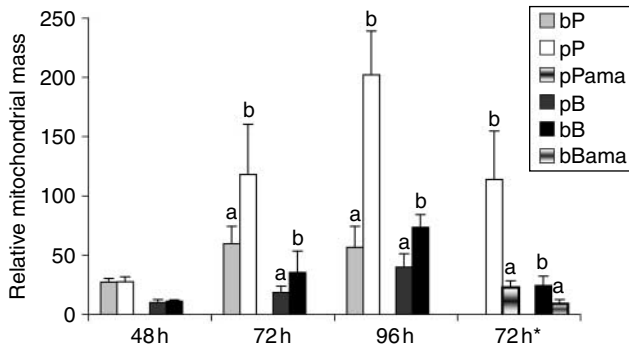


Figure 5 Relative mitochondrial mass (MM) in bovine NT (bB), porcine NT (pP), pig-bovine iSCNT (pB) and bovine-pig iSCNT (bP) embryos stained with JC-1 at 48, 72 and 96 h after activation. At 48 h, MM of NT and iSCNT embryos derived from the same oocytes was comparable. At 72 and 96 h after activation, MM values were significantly higher in NT than iSCNT embryos (a, b: $P < 0.05$). Inhibition of RNA Pol II by α -amanitin at the time of EGA in porcine (pPama) and bovine (bBama) NT embryos led to significant decrease in MM (a, b: $P < 0.05$) at 72 h after activation.

absence of EGA or its aberrant implementation due to nucleus-cytoplasm incompatibility.

Differently from other laboratories (Dominko *et al.* 1999, Uhm *et al.* 2007, Song *et al.* 2008), we did not manage to obtain pig-bovine and bovine-pig iSCNT embryos that could develop to the blastocyst stage, whereas intra-species NT proved to work effectively in our hands. Since the efficiency of NT (Boiani *et al.* 2005, Lagutina *et al.* 2006) and even more of iSCNT (Liu *et al.* 2004, Zhao *et al.* 2006, Sugimura *et al.* 2009) embryo development crucially depends on embryo culture conditions, we tested both SOFaa and CR1aa, which have been previously reported to support bovine (Galli *et al.* 2003) and porcine (Lagutina *et al.* 2006) embryo development and pig-bovine iSCNT blastocyst development (Dominko *et al.* 1999, Uhm *et al.* 2007) respectively. No difference was observed, and pig-bovine/bovine-pig iSCNT embryos were not able to escape the block at the 16–25-cell/four-cell stage, which corresponds to the time of bovine MET (8–16-cell stage; Kanka 2003) and porcine MET (four-cell stage; Tomanek *et al.* 1989, Freitag *et al.* 1991, Jarrell *et al.* 1991). The reasons of such developmental failure in our culture conditions are not clear, but it should not be ascribed to the quality of our *in vitro* maturation-*in vitro* culture (IVC) media or to the NT technique used, since several offspring were born following bovine and porcine NT.

The analysis of remodelling of the donor nucleus in the cytoplasm of a recipient oocyte from a different species indicates that the normal pattern of nucleus remodelling was followed as previously reported in different studies on mammalian NT and iSCNT embryos (Tarkowski & Balakier 1980, Dominko *et al.* 1999, Arat *et al.* 2003, Uhm *et al.* 2007, Lee *et al.* 2008). Thus, the biochemical mechanism of this process is universal, and it works also

in intra-class NT embryos derived from chicken blastodermal cells and rabbit oocytes (Liu *et al.* 2004).

On the other hand, the cleavage kinetics of NT and iSCNT embryos was comparable only up to 48 h of IVC, and during the following 24 h, the development of iSCNT embryos was arrested at the MET stage: that is, at the four-cell stage for bovine-pig embryos and at the 8–16-cell stage for pig-bovine embryos. However, pig-bovine iSCNT embryos divided significantly faster than bovine NT control embryos, and many binucleate blastomeres were observed in about 50% of them. Such accelerated cleavage was reported by Memili & First (1998) in bovine embryos treated with AA to prevent EGA. This finding together with the observation that our iSCNT embryos stopped dividing at the moment of the oocyte MET suggests that EGA did not take place in our iSCNT embryos.

To confirm this hypothesis, we then assessed RNA Pol II expression and activity. Low level of activity was observed in the nuclei of IVF, NT and iSCNT embryos as early as the two-cell stage. This confirms the data on low-grade transcription during the first three cell cycles in bovine embryos (Barnes & First 1991, Plante *et al.* 1994, Hyttel *et al.* 1996, Viuff *et al.* 1996, 1998, Memili & First 1998, Svarcova *et al.* 2007, Kues *et al.* 2008) and extends our knowledge about porcine embryos that were considered transcriptionally inactive until late in the third cell cycle, i.e. at the four-cell stage (Tomanek *et al.* 1989, Freitag *et al.* 1991, Jarrell *et al.* 1991). However, during further IVC, iSCNT embryos did not show the important increase in RNA Pol II activity observed in control embryos, which went through normal EGA.

Moreover, 96 h after activation, iSCNT embryos did not present mature nucleoli. Indeed, immunostaining with an anti-nucleolin antibody revealed faint nucleoplasmic labelling with weakly stained half-rings or rings in bovine nuclei or several dots or rings in some porcine nuclei without formation of mature nucleoli. The same pattern of staining was reported in pig-ovine (Hamilton *et al.* 2004) and rhesus monkey-bovine iSCNT embryos (Song *et al.* 2009) that were not able to properly activate the genome and form BL. Inhibition of RNA Pol I transcription in control NT embryos led to disappearance of nucleolin-labelled mature nucleoli and dispersion of nucleolin into the nucleoplasm. The dispersed nucleoplasmic nucleolin and inability to form nucleoli were also observed in NT embryos after inhibition of EGA as a result of the absence of *de novo* transcription of RNA Pol I that depends on RNA Pol II activity. Therefore, taking into account the developmental block of iSCNT embryos and the absence of RNA Pol II accumulation and its low activity, we conclude that the failure to form mature nucleoli is the result of the absence of EGA.

We also studied the expression pattern of *NANOG* (Brevini *et al.* 2006, Gandolfi *et al.* 2006), which depends on EGA. Porcine *NANOG* mRNA was not

detected even in the best (≥ 16 -cell) pig–bovine iSCNT embryos at the time of porcine and bovine EGA as previously reported for chimpanzee–bovine iSCNT embryos (Wang *et al.* 2009). Comparison of gene expression in 8–16-cell stage human–bovine or human–rabbit embryos and human IVF or NT embryos using single-embryo transcriptome profiling revealed general downregulation of human genes in the bovine and rabbit recipient cytoplasm (Chung *et al.* 2009). These data and the absence of *Alpl2* (*eAp*), *Pou5f1* (*Oct4*) and *Cdh1* (*e-Cad*) expression in mouse embryonic fibroblast (MEF)–bovine iSCNT embryos (Arat *et al.* 2003, Kim *et al.* 2004) suggests that bovine ooplasm does not reprogram properly donor nuclei from other species.

On the other hand, we extended our knowledge about the cytoplasm control in iSCNT embryos over xenonucleus genes using constitutive and tissue-specific inducible promoters. The permanent low RNA Pol II activity in iSCNT embryos was sufficient for the observed GFP accumulation in iSCNT embryos derived from fibroblasts that carry the *GFP* gene under the control of the constitutive *PGK* promoter, as previously described by Uhm *et al.* (2007) using *GFP* controlled by the rat β -actin promoter. This permanent activity of RNA Pol II is enough to sustain *GFP* expression in MEF–bovine (Arat *et al.* 2003) and panda–rabbit iSCNT embryos (Wen *et al.* 2005), avian feather keratin expression in chicken–rabbit intra-classes iSCNT embryos (Liu *et al.* 2004), housekeeping gene expression in mouse–bovine iSCNT embryos (Arat *et al.* 2003, Kim *et al.* 2004). However, the abnormal pattern of housekeeping genes expression (Arat *et al.* 2003, Kim *et al.* 2004, Wang *et al.* 2009) confirmed the inadequate embryonic genome activity in all tested iSCNT embryos.

On the contrary, our data on the absence of transcription of *COL6A1* in iSCNT embryos (Supplementary Figure 5) indicate that bovine cytoplasm can block *de novo* transcription of a fibroblast-specific gene irrespectively of the species affiliation of the donor nucleus. This agrees with the results by Inoue *et al.* (2006) and Green *et al.* (2007) on the silencing of donor cell-specific genes in NT embryos using muscle and hematopoietic cells as nucleus donors and with the statement by Vassena *et al.* (2007) that the donor genome is markedly silenced by the ooplasm at the one-cell stage of NT embryo development. The silencing of porcine genes in bovine cytoplasm does theoretically prove a high homology of elements controlling gene silencing. Conversely, the expression of avian feather keratin in chicken–rabbit intra-class NT embryos (Liu *et al.* 2004) as early as in eight-cell embryos is the example of the inability of mammalian ooplasm to correctly recognise avian tissue-specific inducible promoters or silencers.

Another interesting question of iSCNT embryo development is the interaction of the xeno-nucleus with the cytoplasmic mitochondria that have their own DNA and can synthesise RNAs and proteins. It is known

that the number of mtDNA copies/embryo is thought to be stable during cleavage until replication begins at an undefined time post-implantation (Aiken *et al.* 2008) and the total number of mitochondria does not increase during the preimplantation stage of development (Szollosi 1965). However, during early embryo development, mitochondria undergo changes in shape and structure and the number of cristae increases (Betteridge 1988, Van Blerkom 2009). The detailed study (Mtango *et al.* 2008) of temporal expression of mRNAs related to mitochondrial biogenesis revealed a persistent expression of maternally encoded mRNAs in combination with transcriptional activation and mRNA accumulation around the time of EGA. All proteins involved in mitochondrial biogenesis and functions, currently estimated at ~ 1500 , are encoded by nuclear DNA, synthesised in the cytosol, and then targeted and imported into mitochondria by specific mechanisms (Cotter *et al.* 2004, Hiendleder *et al.* 2005). Lee *et al.* (2002) demonstrated that the increase in mitochondrial mass does not depend on mtDNA presence or function but on cytoplasmic protein synthesis. Thus, changes of mitochondrial mass in embryos are most probably dependent on EGA. We have found that the relative mitochondrial mass and J-aggregate content were comparable in NT and iSCNT embryos at 48 h of IVC when only maternally inherited transcripts were available. However, both parameters in iSCNT embryos at 72 h post-activation were significantly lower than in control NT embryos and resembled those of NT embryos after inhibition of EGA. These results further confirm that EGA is not taking place in iSCNT embryos, and suggest that comparison of the relative mitochondrial mass in iSCNT and control NT embryos at the time of the host oocyte's EGA can serve as an express test to assess genome activation in iSCNT embryos. Another interesting question of the interaction of the xeno-nucleus with the mitochondria in iSCNT embryos that was not studied here is the level of compatibility between nuclear and mitochondrial genomes in iSCNT embryos as the existence of species-specific incompatibility between nuclear and mitochondrial genomes of closely related species has been demonstrated in cybrids of primates (Kenyon & Moraes 1997, Barrientos *et al.* 2000) and murids (Dey *et al.* 2000, McKenzie & Trounce 2000, McKenzie *et al.* 2003).

To summarise, our data strongly indicate that bovine and porcine oocytes cannot properly reprogram the genome of each other in iSCNT embryo and activate embryonic genome transcription at the time of MET. The causes of such species incompatibility should be investigated more carefully. One of the reasons might be some specific differences in gene structure. The high homology of mammalian proteins and their coding genes in the case of iSCNT seems not to guarantee proper gene function. For instance, the mouse *Pou5f1* promoter correctly activates *GFP* transcription at the

time of EGA in bovine NT embryos derived from bovine fibroblasts carrying a mouse *Pou5f1* promoter-driven *GFP* reporter gene (Wuensch *et al.* 2007), while it does not in porcine NT embryos derived from porcine fibroblasts carrying the same transgene (D Brunetti, personal communication). An aberrant DNA methylation pattern in iSCNT embryos may be another reason. Chen *et al.* (2004) demonstrated that demethylation of donor repetitive sequences is driven by the recipient ooplasm, and ooplasm from different species have different capabilities to demethylate genes. The results of our study raise the question of inter-species nuclear–cytoplasmic or genomic compatibility. The role of nuclear–cytoplasmic interactions in EGA, gene activation and dependence of the activation of a given gene on the function of others is a large field that needs to be investigated.

Materials and Methods

Oocyte maturation

Unless otherwise stated, all chemicals were purchased from Sigma–Aldrich. Oocytes were aspirated from 3 to 8 mm in diameter ovarian follicles of killed animals. *In vitro* manipulation was carried out essentially as described previously (Lagutina *et al.* 2006). Bovine oocytes were matured in TCM199 supplemented with 10% (v/v) FCS, 1 µl/ml ITS Media Supplement (insulin, transferrin, selenite, Sigma, cat. no. I1884), 1 mM sodium pyruvate, 0.5 mM L-cysteine, 10 mM glycine, 100 µM β-mercaptoethanol, gonadotropins (0.05 IU/ml FSH and 0.05 IU/ml LH; Pergovet 75, Serono). Porcine oocytes were matured in DMEM-F12 supplemented with 10% (v/v) FCS, 110 µg/ml sodium pyruvate, 75 µg/ml ascorbic acid, 100 µg/ml glutamine, 5 µg/ml myoinositol, 0.4 mM cysteine, 0.6 mM cysteamine, ITS Media Supplement (1 µl/ml), gonadotropins (0.05 IU/ml FSH and 0.05 IU/ml LH; Pergovet 75, Serono), 100 ng/ml LONG R3 insulin-like growth factor 1 (IGF1; recombinant IGF analogue), 50 ng/ml LONG epidermal growth factor (EGF; recombinant EGF analogue) and 5 ng/ml bovine fibroblast growth factor (bFGF; human recombinant). Bovine and porcine oocytes were cultured at 38.5 °C in 5% CO₂ in humidified air for 21 and 42 h respectively.

Production of bovine and porcine IVF embryos

Bovine IVF embryos were produced as previously described (Lagutina *et al.* 2002). Porcine IVF embryos: after 42 h of maturation, oocytes were partially denuded of the cumulus cells and cultured in four-well plates containing 0.3 ml/well of TALP prepared without glucose, supplemented with 1 mg/ml heparin, 20 mM D-penicillamine, 100 mM hypotaurine, 1 mM adrenaline, and 0.1×10^6 spermatozoa/ml. Motile spermatozoa were obtained by centrifugation of frozen–thawed semen on a Percoll discontinuous density gradient for 40 min at 750 *g*, washed in Ca-free TALP and pelleted by centrifugation for 10 min at 400 *g*.

Preparation of donor cells

Bovine and porcine adult and foetal fibroblasts and porcine foetal GFP-positive fibroblasts (*GFP* under the control of the *PGK* promoter) at passages 1–10 were used as donor cells. Fibroblasts were derived from ear biopsies according to standard procedures and cultured in DMEM+TCM 199=1:1 with 10% FCS. The medium for porcine fibroblasts was supplemented with 5 ng/ml bFGF (human recombinant). All donor cells were tested for efficient blastocyst development after intra-species NT. Two days before NT, donor cells were passaged, and one day before NT, they were serum starved (0.5% FCS) for 24 h.

Nuclear transfer

NT was carried out essentially as described previously (Lagutina *et al.* 2006). Briefly, oocytes were denuded of cumulus cells by vortexing in SOF-HEPES in the presence of hyaluronidase, and oocytes with extruded polar bodies were selected. NT embryos were reconstructed following a zona-free method (Oback *et al.* 2003). After zona pellucida digestion with 0.5% pronase in PBS, oocytes were washed in SOF-HEPES with 10% FCS and returned to maturation medium. All further manipulations were performed in SOF-HEPES with 10% FCS. Zona-free oocytes were exposed to cytochalasin B (7.5 µg/ml) and Hoechst (5 µg/ml) for 5 min. Metaphase chromosomes were removed under u.v. light with a blunt nucleation pipette. Then, each cytoplasm was individually washed for few seconds in 300 µg/ml phytohemagglutinin P in PBS and quickly dropped over a single donor cell (Vajta *et al.* 2003) settled at the bottom of a microdrop of donor cell suspension diluted in SOF-HEPES. Formed cell couples were washed in 0.3 M mannitol (Ca⁺⁺-free, 100 µM Mg⁺⁺) solution, fused by double DC-pulse of 1.2 kV/cm applied for 30 µs and returned to maturation medium.

Activation

Bovine oocytes

Embryos (2–3 h after fusion) were activated 27–28 h after the onset of oocyte maturation with 5 µM ionomycin in SOF-HEPES for 4 min followed by 4-h culture in individual 2-µl drops of 2 mM 6-dimethylaminopurine in SOF, supplemented with MEM essential and non-essential amino acids and 4 mg/ml BSA (m-SOFaa) at 38.5 °C in humidified atmosphere of 5% CO₂, 5% O₂ and 90% N₂.

Porcine oocytes

Embryos (2–3 h after fusion) were activated 48–50 h after the onset of oocyte maturation by double DC-pulses of 1.2 kV/cm for 30 µs in 0.3 M mannitol solution, containing 1 mM Ca⁺⁺ (Cheong *et al.* 2002) and 100 µM Mg⁺⁺, followed by 4-h culture in maturation medium with 5 µg/ml cytochalasin B at 38.5 °C in humidified atmosphere of 5% CO₂, 5% O₂ and 90% N₂.

Embryo in vitro culture

NT and iSCNT embryos were cultured according to the WOW method (Vajta *et al.* 2000) individually in microwells in 20- μ l drops of m-SOFaa in 5% CO₂, 5% O₂ and 90% N₂ humidified atmosphere at 38.5 °C. Half of the medium was renewed with fresh m-SOFaa at days 4 and 6 (day 0 was the day of NT). Cleavage was assessed 48 h after activation, the occurrence of BL at day 6 and 7. All experiments were done using three or more replicates (unless otherwise indicated). However, some experiments were done in CR1 medium supplemented with MEM essential and non-essential aminoacids that were claimed to be efficient for the production of iSCNT BL in several species using bovine oocytes (Dominko *et al.* 1999, Uhm *et al.* 2007).

To assess the effect of EGA inhibition on the formation of nucleoli, porcine and bovine embryos were cultured in medium supplemented respectively with 25 μ g/ml AA or 0.2 μ g/ml AD in the window of time going from 48 to 96 h of IVC.

To assess the effect of inhibition of RNA Pol I activity on the function of nucleoli, bovine and porcine embryos were cultured in medium supplemented with 0.2 μ g/ml AD for 3 h starting at 93 h of IVC.

To evaluate the effect of inhibition of EGA on mitochondria, bovine and porcine NT embryos were cultured in medium supplemented with 25 μ g/ml AA starting at 48 h and till 72 h after activation.

RT-PCR

Unless otherwise indicated, all chemicals were purchased from Invitrogen.

RNA extraction from pig–bovine iSCNT embryos

Poly(A)⁺ RNA was isolated from three separate groups of five iSCNT embryos at the 2-, 4-, 8- and 16-cell stage using the Dynabeads mRNA direct MicroKIT (Dyna-Invitrogen), following the manufacturer's instruction (Supplementary Materials and Methods, see section on supplementary data given at the end of this article).

RT

For each sample, RT was carried out in a total volume of 20 μ l of reaction mixture using 1 μ l of Oligo(dT)_{12–18} primer and 1 μ l dNTP Mix (10 mM each). RNA was denatured at 65 °C for 5 min, then 4 μ l of first-strand buffer (250 mM Tris–HCl pH 8.3, 375 mM KCl and 15 mM MgCl₂), 1.5 μ l 50 mM MgCl₂, 2 μ l of 0.1 M dithiothreitol and 1 μ l of RNaseOUT Recombinant Ribonuclease Inhibitor (40 U/ μ l) were added. RT was performed with 200 U (1 μ l) Superscript II RT for 1 h at 42 °C, followed by a denaturation step at 70 °C for 15 min. Tubes without RNA or RT were prepared as negative controls (data not shown).

PCR

Accession number, primer sequences, annealing temperature, amplification product sizes and number of amplification cycles for each gene are presented in Supplementary Table 4, see

section on supplementary data given at the end of this article. Rabbit globin mRNA was used as exogenous standard to normalise reactions for the amount of RNA in each sample. PCR amplification was performed with cDNA concentrations equivalent to one embryo from different pools of iSCNT embryos. The reaction mix consisted of 11 μ l of 50 mM MgCl₂, 0.3 μ l of Taq DNA polymerase (5 U/ μ l), 2 μ l of 10 \times PCR buffer (200 mM Tris–HCl pH 8.4, 500 mM KCl), 1 μ l NTP Mix (10 mM each), 1 μ l (100 pmol) of each sequence-specific primer and sterile water up to 20 μ l. Amplifications were carried out in an automated thermal cycler (iCycler, Bio-Rad). To confirm the identity of the RT-PCR fragments, amplification products from each primer pair were separated by gel electrophoresis in 2% agarose gels, purified using Spin-X centrifuge tube filters (Corning, Milan, Italy; Sigma–Aldrich), sequenced (SEQLAB, Gottingen, Germany) and aligned using Clustal W 1.82 (EMBL–EBI service).

Immunocytochemistry

IVF, NT and iSCNT embryos at 24, 48, 72 and 96 h after activation were fixed in 4% paraformaldehyde in PBS with 0.1% Triton X-100 at 4 °C for 3 h and subsequently stained with antibodies against nucleolin (C23, an indirect marker of RNA Pol I activity; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA, sc-8031) and RNA Pol II (CTD4H8; Covance Research Products Inc., Princeton, NJ, USA) overnight. Embryos were washed in PBS/BSA and incubated with the secondary antibody conjugated with FITC (Santa Cruz Biotechnology) at room temperature for 2 h, washed in PBS/BSA and mounted in Cytifluor with DAPI.

FISH

RNA Pol II activity was assessed in IVF, NT and iSCNT embryos at 24, 48, 72 and 96 h after activation by detection of polyadenylated transcripts in the nuclei using RNA FISH with a fluorescein-labelled oligo dT probe (Sigma). This probe was 45 bases long with fluorescein inserted every ten position. The method was based on the protocol by Tam *et al.* (2002) with slight modifications for embryos (Supplementary Materials and Methods).

Analysis of GFP reporter gene expression

To visualise RNA Pol II activity in pig–bovine iSCNT embryos derived from porcine foetal fibroblasts that carry the *GFP* reporter gene under the control of the constitutive promoter *PGK*, we estimated the expression of the *GFP* gene 24 h after activation and at day 7 under a fluorescence microscope using a standard FITC filter set.

Assessment of mitochondrial changes

We used JC-1 fluorescent dye (Invitrogen) that accumulates in mitochondria to assess the behaviour of mitochondria in bovine and porcine NT and iSCNT embryos. Embryos at 48, 72 and 96 h after activation were stained with 2 μ M JC-1 in SOF-HEPES with 10% FCS at 37 °C for 1 h. Images were collected using a double FITC/Rodamine filter. Both red and

green fluorescence emissions from the area of the embryo where dye distribution artefacts have been removed were analysed with Adobe Photoshop Elements 2. The data were presented as a mean fluorescence intensity of the embryo. In total, not less than 20 embryos per every group/time point were analysed in three replicates.

Nucleus remodelling, number of nuclei and cells

NT and iSCNT embryos 1 h after fusion and 3, 48, 72 and 96 h after activation were stained with Hoechst 33342 to estimate morphology, number and distribution of nuclei.

ApoAlert Annexin V staining

Staining of NT and iSCNT embryos at 96 h after activation was performed according to the manufacturer's instructions (Clontech Laboratories, Inc). Images were analysed using FITC and Rodamine filters.

Images

Embryos were viewed with the fluorescence microscope Nikon Eclipse 80i using FITC, Rodamine or dual filter set for FITC/Rodamine, and images were collected using a DS Camera Head DS-5M with DS Camera Control Unit DS-L1 (Nikon Instruments, Milan, Italy).

Statistical analysis

Differences between the experimental groups were verified using Student's *t*-test and the χ^2 test. A value of $P < 0.05$ was considered significant.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-09-0578>.

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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