

Formation of nucleoli in interspecies nuclear transfer embryos derived from bovine, porcine, and rabbit oocytes and nuclear donor cells of various species

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Abstract

The most successful development of interspecies somatic cell nuclear transfer (iSCNT) embryos has been achieved in closely related species. The analyses of embryonic gene activity in iSCNT embryos of different species combinations have revealed the existence of significant aberrations in expression of housekeeping genes and genes dependent on the major embryonic genome activation (EGA). However, there are many studies with successful blastocyst (BL) development of iSCNT embryos derived from donor cells and oocytes of animal species with distant taxonomical relations (inter-family/inter-class) that should indicate proper EGA at least in terms of RNA polymerase I activation, nucleoli formation, and activation of genes engaged in morula and BL formation. We investigated the ability of bovine, porcine, and rabbit oocytes to activate embryonic nucleoli formation in the nuclei of somatic cells of different mammalian species. In iSCNT embryos, nucleoli precursor bodies originate from the oocyte, while most proteins engaged in the formation of mature nucleoli should be transcribed from genes *de novo* in the donor nucleus at the time of EGA. Thus, the success of nucleoli formation depends on species compatibility of many components of this complex process. We demonstrate that the time and cell stage of nucleoli formation are under the control of recipient ooplasm. Oocytes of the studied species possess different abilities to support nucleoli formation. Formation of nucleoli, which is a complex but small part of the whole process of EGA, is essential but not absolutely sufficient for the development of iSCNT embryos to the morula and BL stages.

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Introduction

The formation of ruminant-type nucleolus precursor bodies (NPBs) in interspecies somatic cell nuclear transfer (iSCNT) embryos derived from porcine donor cells and ovine oocytes has been demonstrated by means of transmission electron microscopy (Hamilton *et al.* 2004). Hamilton *et al.* suggested that 'the ovine ooplasm is having a profound influence on the formation of the NPB in the porcine nucleus'. This interesting observation was explained by the demonstration of the maternal origin and inheritance of nucleoli (Ogushi *et al.* 2008). Ogushi *et al.* experimentally proved that porcine and murine embryos failed to develop if the oocytes were enucleolated at the germinal vesicle stage and that fertilized/SCNT embryos restored their developmental ability after reinjection of isolated oocyte nucleoli at the MII stage. Nucleoli originating from fibroblast or embryonic stem cell nuclei were not able to substitute

maternal nucleoli and support the development of NT embryos derived from enucleolated oocytes. The nature of these indispensable maternally inherited factors of nucleoli is unknown. In intra-species SCNT embryos, there is compatibility between the autologous cytoplasm, nucleus, and nucleoli. In contrast, iSCNT embryos are composed of maternally inherited cytoplasm, a xeno-nucleus, and NPBs originating from the oocyte. For successful embryo development, cytoplasmic factors should correctly reprogram and properly activate the genome of the xeno-nucleus.

In *Xenopus laevis*, it was found that major embryonic genome activation (EGA) initiated with activation of class II genes (mRNA), followed by class III genes (tRNA), and finally class I genes (rRNA; Shiohara *et al.* 1989, 1994). In mammals, this was confirmed by the demonstration that RNA polymerase I as well as other key nucleolar proteins, upstream binding factor (UBF) and topoisomerase I, engaged in transcription of the

rDNA, and fibrillarin (Svarcova *et al.* 2007), which is involved in early processing of rRNA, appeared in embryos at the time of EGA (Maddox-Hyttel *et al.* 2007, Svarcova *et al.* 2007). The rRNA gene activation and the associated nucleolus formation are suggested as markers of the major EGA in mammalian embryos (Bjerregaard *et al.* 2007). The activation of rDNA transcription begins from the attachment of UBF to the upstream control sequence (UCS) and formation of the RNA polymerase I complex on the promoter. UCS recruits and binds a protein complex incorporating TATA-binding protein (TBP) and three TBP-associated factors (TAFs) called SL1 (human) or TIF-IB (mouse). Transcription starts after polymerase I binding to the UBF/SL1 complex via phosphorylated RRN3/TIF-IA (Russell & Zomerdijk 2005, 2006). The impaired pre-rRNA transcription (Baran *et al.* 2003, Chen *et al.* 2008) or the inhibition of polymerase I transcription (Baran *et al.* 2003) led to fragmentation of nucleoli, apoptotic nuclei, and decreased cell proliferation prior to the morula stage. Similar phenotypes of preimplantation lethality before the blastocyst (BL) stage were observed in knockout mice or in knockdown embryos in the case of genes involved in ribosome biogenesis: Pescadillo (Lerch-Gaggl *et al.* 2002), fibrillarin (Newton *et al.* 2003), and SURF6 (Romanova *et al.* 2006). Thus, the inability of iSCNT embryos to correctly activate, transcribe, and translate genes involved in pre-rRNA synthesis at the time of EGA may cause the arrest of nucleoli formation and developmental block in embryos. The high level of gene similarity between mammalian species does not guarantee inter-species compatibility of their products. It was shown (Heix *et al.* 1997) that the primary structure of human and mouse TAFs does not dramatically alter the network of protein-protein contacts responsible for assembly of the multimeric complex SL1/TIF-IB. The primate versus rodent promoter selectivity mediated by the TBP-TAFs complex is likely to be the result of cumulative subtle differences between individual subunits that lead to species-specific properties of RNA polymerase I transcription.

The absence of mature nucleoli can indirectly indicate silencing or aberrant expression of genes encoding RNA polymerase I, nucleolar proteins, and, as a result, rRNA genes.

Nucleolin is a maternally inherited (Rickards *et al.* 2007, Svarcova *et al.* 2007) multifunctional nucleolar protein that has been implicated in chromatin structure, rDNA transcription, rRNA maturation, early stages of ribosome assembly, and nucleo-cytoplasmic transport (Ginisty *et al.* 1999). Immunofluorescence microscopy confirmed that nucleolin localizes primarily to nucleoli with RNA polymerase I. Ultrastructural studies of nucleoli showed main localization of nucleolin in the dense fibrillar component (DFC) around fibrillar centers. However, a small portion has also been detected in the granular component, but it is rarely seen in the fibrillar

centers. Nucleolin binds preferentially to a DNA fragment containing the region of the non-transcribed spacer of rDNA located upstream of the site of transcription initiation (Olson *et al.* 1983). Along with the ability of nucleolin to interact with histone H1 and to modulate chromatin structure (Erard *et al.* 1988, 1990), this suggests that nucleolin could be involved in the regulation of rRNA transcription. Since nucleolin was not found in the polymerase I initiation complex, it was proposed that it does not play a direct role in transcription initiation (Ginisty *et al.* 1999). However, the specific inhibition of RNA polymerase I transcription after nucleolin knockdown by RNA interference (Rickards *et al.* 2007) showed that an important function of nucleolin is to permit RNA polymerase I to transcribe nucleolar chromatin (Rickards *et al.* 2007). Indeed, following inhibition of rRNA transcription, a rapid release of nucleolin from the DFC of the nucleolus was observed (Escande-Geraud *et al.* 1985), demonstrating that its presence in this subnucleolar compartment was dependent upon rRNA transcription. Owing to high structural homology (Ginisty *et al.* 1999), nucleolin can be detected by C23 antibody labeling in many mammalian species. All these characteristics make nucleolin an ideal marker of nucleoli.

The aim of our research was to evaluate the ability of bovine, porcine, and rabbit oocytes to support nucleoli formation in iSCNT embryos derived from bovine, buffalo, ovine, porcine, equine, rabbit, canine, feline, and mouse nuclear donor cells, and to estimate the developmental ability of iSCNT embryos with activated rRNA synthesis.

Results

Nucleolin (C23) labeling of nucleoli in adult fibroblasts of different species

Anti-C23 antibody labeling of nucleolin permitted visualization of active nucleoli in the nuclei of bovine, buffalo, ovine, equine, mouse, porcine, canine, feline, and rabbit adult fibroblasts (Fig. 1).

Nucleolin labeling of bovine and porcine IVF and SCNT embryos at the time of genome activation

To characterize the formation of nucleoli, we labeled bovine (Fig. 2) and porcine (Fig. 3) IVF and SCNT embryos with anti-C23 antibody at 48, 72, and 96 h after IVF/activation.

Bovine embryos

At 48 h post IVF/activation, the most advanced embryos were at the 8-cell stage. Anti-C23 labeling (Fig. 2) revealed homogenous nucleoplasmic localization of nucleolin with concentration in several dots or shells in some nuclei. At 72 h post IVF/activation (8- to 16-cell

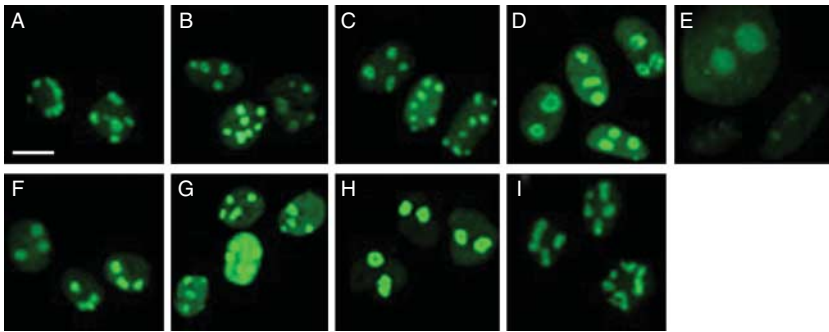


Figure 1 Anti-nucleolin (C23) labeling of nucleoli in the nuclei of (A) bovine; (B) buffalo; (C) ovine; (D) equine; (E) mouse; (F) porcine; (G) canine; (H) feline; and (I) rabbit adult fibroblasts. Scale bar: 10 μ m.

stage), nucleolin staining in IVF and SCNT embryos became more intense and appeared in the form of shell-like structures indicating the formation of nucleoli. At 96 h, embryos possessed 16 or more cells, with the most advanced embryos forming compacting morulae. Mature nucleoli were formed and appeared as intensively labeled polygonal structures. During nucleologenesis, most nucleolin from nucleoplasm relocated to the nucleoli (Fig. 2).

Porcine embryos

At 48 h post IVF/activation (4-cell stage), nucleoplasmic nucleolin labeling (Fig. 3) was concentrated around presumptive NPBs in the form of shell-like structures.

During subsequent culture (72–96 h) in IVF and SCNT embryos, the intensity of nucleolin staining of shell-like structures was significantly increased, and at 96 h (four and more cells) intensively, nucleolin-positive mature nucleoli were visible. The formation of mature nucleoli was accompanied by decreasing of nucleolin labeling in the nucleoplasm (Fig. 3).

Evaluation of nucleoli formation in iSCNT embryos and their development

The development and results of nucleolin staining of iSCNT embryos derived from donor cells of various species and bovine (Figs 4 and 5), porcine (Figs 6 and 7), and rabbit (Fig. 8) oocytes are shown in the Table 1.

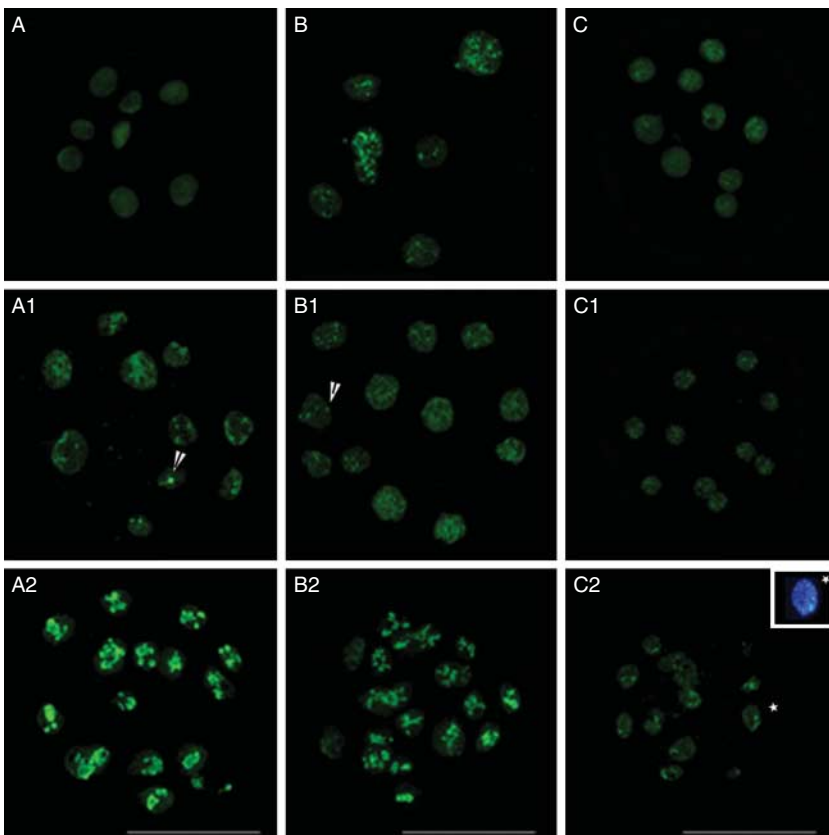


Figure 2 Anti-nucleolin (C23) labeling of the most advanced bovine IVF (A, A1, and A2), SCNT (B, B1, and B2), and porcine-bovine iSCNT (C, C1, and C2) embryos at 48 (A, B, and C), 72 h (A1, B1, and C1), and 96 h (A2, B2, and C2) after fertilization/activation. C23 labeling revealed nucleoplasmic nucleolin distribution at 48 h, and the formation of dot-like nucleolin-labeled structures (arrow heads) at 72 h in IVF and SCNT embryos. Polygonal mature nucleoli in the nuclei of IVF (A2) and SCNT (B2) embryos, and the absence of mature nucleoli in porcine-bovine iSCNT (C2) embryos at 96 h after fertilization/activation. DAPI counterstaining highlighted chromatin condensation in the nucleus of iSCNT embryo. Scale bars, 100 μ m.

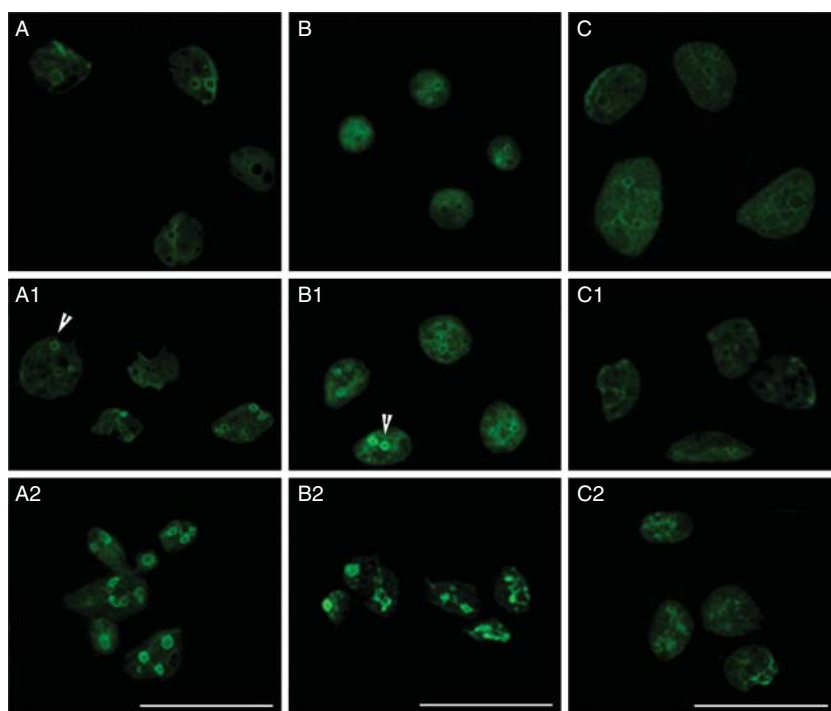


Figure 3 Anti-nucleolin (C23) labeling of the most advanced porcine IVF (A, A1, and A2), NT (B, B1, and B2), and bovine-porcine iSCNT (C, C1, and C2) embryos at 48 (A, B, and C), 72 h (A1, B1, and C1), and 96 h (A2, B2, and C2) after fertilization/activation. C23 labeling revealed nucleoplasmic nucleolin staining at 48 h, and the formation of ring-like nucleolin-labeled structures (arrow heads) at 72 h in IVF and SCNT embryos. Shell-like mature nucleoli in the nuclei of IVF (A2) and SCNT (B2) embryos, and the absence of mature nucleoli in porcine-bovine iSCNT (C2) embryos at 96 h after fertilization/activation. Scale bars, 100 μ m.

Bovine oocytes

Bovine cytoplasm efficiently supported the development of iSCNT embryos to the 8- to 16-cell stage (the stage of bovine EGA) irrespective of donor cell species affiliation. The kinetics of early cleavage for iSCNT embryos was comparable with that for bovine embryos. iSCNT embryos derived from porcine (Fig. 2), equine, rabbit, feline, canine, and mouse (Fig. 5B–F respectively) cells failed to form nucleoli 96 h after activation. These iSCNT embryos with bovine cytoplasm possessed prevailing nucleoplasmic nucleolin localization with occasional concentration in several faintly stained dots or shells in some nuclei. Their development was blocked at the stage of bovine EGA. The absence of EGA resulted in chromatin condensation in most of the nuclei (Figs 2C2 and 5C–E).

We found the formation of nucleoli in ovine nuclei (Fig. 5) of ovine-bovine iSCNT embryos that might suggest partial genome activation. However, while 35% of ovine-bovine iSCNT embryos developed *in vitro* to the 12- to 16-cell stage on day 4, none of the 247 embryos were able to compact and form BLs during further culture (Table 1).

We found large shell-like mature nucleoli in buffalo nuclei 96 h after activation (Fig. 4B). Interestingly, they appeared similar to the buffalo nucleoli in buffalo SCNT embryos (Fig. 4A) in contrast to branching polygonal nucleoli of control bovine IVF and SCNT embryos at the same time point (Fig. 2A2 and B2). The presence of active RNA polymerase I in nucleoli of buffalo-bovine iSCNT embryos (Fig. 4C) was confirmed with the actinomycin D (AD) test. Short (3 h) low dose (0.02 μ g/ml) AD inhibition of RNA polymerase I activity on day 4 resulted in complete disappearance of C23-labeled nucleoli and nucleolin translocated into the nucleoplasm. Some buffalo-bovine embryos (Table 1) were able to start the fifth cell cycle and even compaction indicating buffalo EGA. There was an effect of donor cell type on the development of buffalo-bovine iSCNT embryos. About 10% of day 6 buffalo-bovine iSCNT embryos derived from granulosa cells reached the 20- to 27-cell stage, however, without any sign of compaction. In contrast, about 3% of buffalo-bovine embryos derived from adult fibroblasts were on compacting morula stage and possessed large polygonal mature nucleoli.

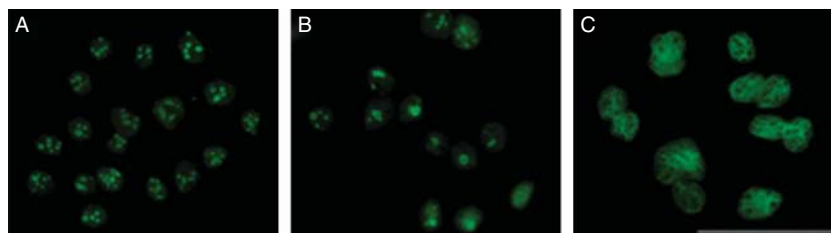


Figure 4 Advanced buffalo SCNT (A) and buffalo-bovine iSCNT (B and C) embryos at 96 h after activation. Distinct nucleolar localization of C23 in mature nucleoli in buffalo SCNT (A) and buffalo-bovine iSCNT (B) embryos and disappearance of functional nucleoli from the nuclei of buffalo-bovine iSCNT (C) embryos after 3 h treatment with 0.2 μ g/ml AD. Scale bar: 100 μ m.

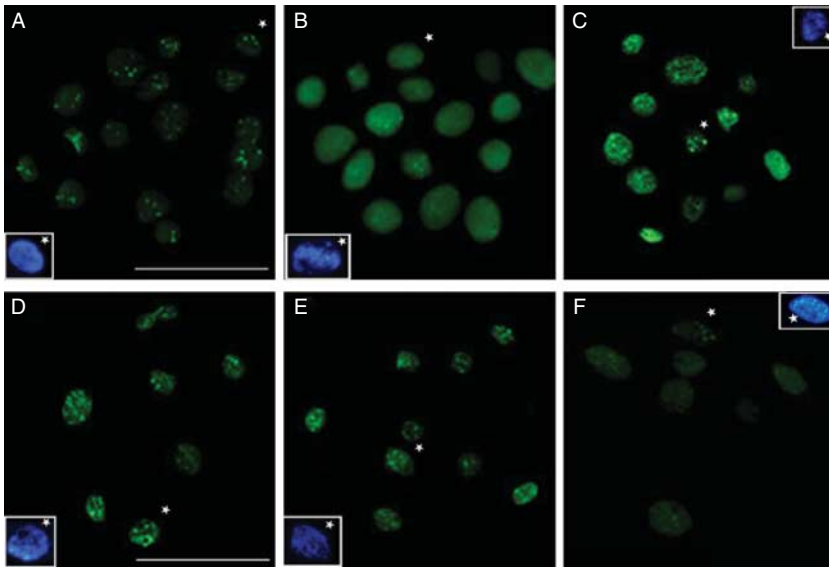


Figure 5 Formation of dot-like anti-nucleolin-labeled structures in ovine–bovine iSCNT embryos (A) and the absence of mature nucleoli in equine (B), rabbit (C), feline (D), canine (E), and mouse (F) iSCNT embryos derived from bovine oocyte at 96 h after activation. DAPI counterstaining highlighted the presence of homogeneously stained nuclei in ovine–bovine iSCNT embryos (A) and chromatin condensation in the nuclei of iSCNT embryos derived from equine (B), rabbit (C), feline (D), canine (E), and mouse (F) nuclear donor cells. Scale bar: 100 μ m.

Porcine oocytes

The kinetics of early cleavage of iSCNT embryos with porcine cytoplasm, irrespective of donor cell species affiliation, was comparable to that of porcine embryos that possess long 4-cell stage (more than 48 h) – the stage of porcine EGA. Anti-C23 staining on day 4 revealed that bovine (Fig. 3C2), buffalo, and mouse nuclei (Fig. 7A and F respectively) failed to form nucleoli. These iSCNT embryos with porcine cytoplasm exhibited prevailing nucleoplasmic nucleolin localization with occasional concentration around NPBs. Their development was blocked at the 4- to 6-cell stage.

We found shell-like nucleoli in porcine nuclei, typical of intra-species porcine SCNT embryos (Figs 3B2 and 6A), and in equine, rabbit, feline, and canine nuclei in iSCNT embryos (Figs 6B and 7C–E respectively) with porcine cytoplasm on day 4. The development of rabbit–porcine and equine–porcine iSCNT embryos (Table 1), which formed large mature nucleoli with active RNA polymerase I (AD test; Fig. 6B1), was blocked at 4- to 8-cell stages. However, nucleoli were observed in some nuclei of such iSCNT embryos on day 6. Formation of shell-like anti-nucleolin-labeled structures in ovine–porcine iSCNT embryos (Fig. 7B) suggested initiation of nucleoli formation. However, the size, the intensity of nucleolin staining, and the thickness of shell-like structures suggested incompleteness of the process or their immaturity.

Rabbit oocytes

Rabbit SCNT embryos display relatively rapid cleavage, and at 72 h, about 22% of embryos initiated the fifth cell cycle and reached the stage of compacting morula or early BL. The delayed embryos possessed 8 to 20 nuclei. All rabbit SCNT embryos with eight and more nuclei

72 h after activation possessed mature nucleoli (Fig. 8A). Analyses of iSCNT embryos at 72 h after activation showed that rabbit cytoplasm efficiently supported initial development of iSCNT embryos to the 4-cell stage (porcine–rabbit iSCNT, 44%; cattle–rabbit iSCNT, 81%). Bovine–rabbit iSCNT embryos were able to start the fourth cell cycle and reached the 9- to 16-cell stage at 72 h after activation. This coincided with the development of advanced bovine embryos at the time of EGA

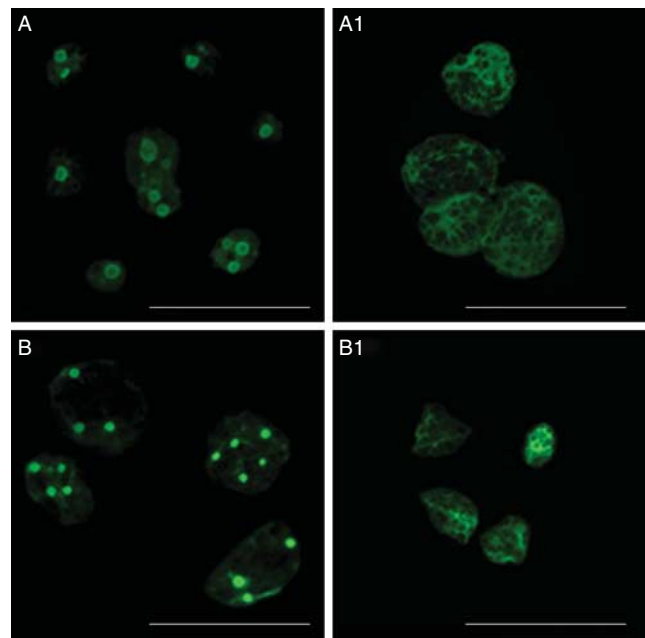


Figure 6 Porcine SCNT (A and A1) and equine–porcine iSCNT (B and B1) embryos at 96 h after activation. Nucleolin localization (C23) in mature nucleoli of porcine SCNT (A) and equine–porcine iSCNT (B) embryos, disappearance of mature nucleoli, and dispersed nucleoplasmic nucleolin labeling in actinomycin D-treated porcine SCNT embryos (A1) and equine–porcine iSCNT (B1) embryos. Scale bar: 100 μ m.

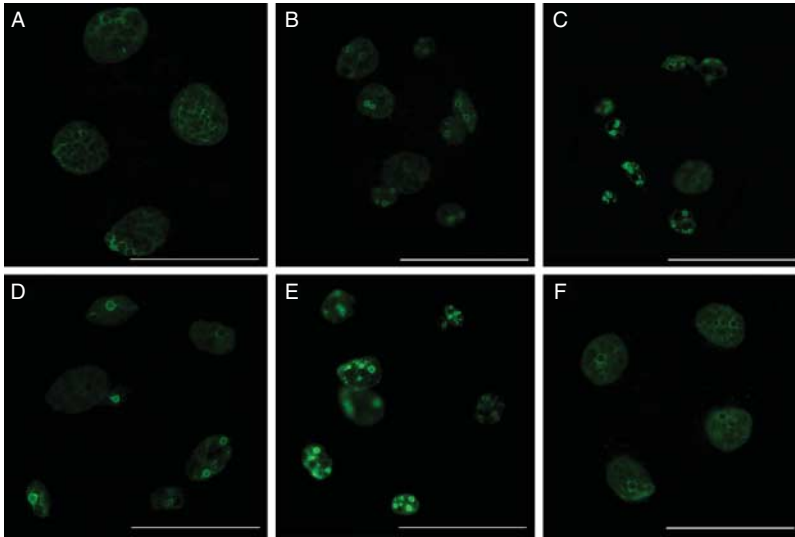


Figure 7 Formation of ring-like anti-nucleolin-labeled structures in ovine-porcine iSCNT embryos (B) and nucleoli in rabbit (C), feline (D), and canine (E) iSCNT embryos derived from porcine oocyte at 96 h after activation. Absence of nucleoli in (A) buffalo-porcine iSCNT embryos and (F) mouse-porcine iSCNT embryos. Scale bar: 100 μ m.

and of delayed rabbit SCNT embryos after EGA. Anti-C23 staining revealed numerous nucleoli of different sizes in almost half of these embryos (Fig. 8B). As in bovine IVF/SCNT embryos, mature nucleoli appeared only on day 4; thus, it is evident that the time of nucleoli formation was determined by rabbit cytoplasm. The formation of nucleoli indicated at least partial bovine genome activation.

The development of porcine-rabbit iSCNT embryos was completely blocked at the time of porcine (four cells)/rabbit (four to eight cells) EGA with five to nine nuclei in the most advanced embryos. Almost all nuclei of porcine-rabbit iSCNT embryos were blocked at the stage of mitotic chromosome condensation when genome is silent. We did not find any sign of nucleoli formation (Fig. 8C) even in the most advanced embryos with eight to nine nuclei. The level of nucleoplasmic nucleolin was also low.

Discussion

Studying the process of nucleoli formation in control IVF and SCNT bovine and porcine embryos revealed that the time of EGA, indicated by the formation of nucleoli in both species, was almost identical despite the difference in the

stage of EGA (bovine: 8- to 16-cell stage; porcine: 4-cell stage). Hence, at 96 h, mature active nucleoli were present in all developmentally competent control embryos. Rabbit SCNT embryos, which develop faster than porcine and bovine embryos, formed nucleoli much earlier, and by 72 h of IVC (the time of morula/BL), all embryos with eight and more blastomeres possess mature nucleoli.

We found differences in the capacities of bovine and porcine oocytes to support nucleoli formation in xenonuclei. Bovine oocytes were able to support nucleoli formation in the nuclei of only closely related species such as water buffalo (inter-genus) and domestic sheep (inter-subfamily) that have very similar kinetics of embryo cleavage, time, and stage of EGA and likely a similar structure of NPBs. Ovine and bovine embryos form ruminant-type NPBs (Hamilton *et al.* 2004). There are no electron microscopical data on the type of NPBs in buffalo embryos. However, the inter-genus relationship of buffalo and bovine species is closer than the inter-subfamily relationship between ovine and bovine. The similar polygonal and tubular form of nucleolin-labeled active mature buffalo nucleoli predicted a ruminant type of NPBs.

We observed the formation of large intensively nucleolin-labeled nucleoli in buffalo-bovine iSCNT

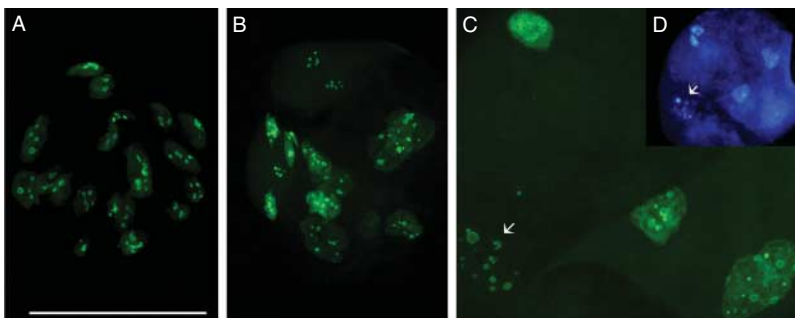


Figure 8 Anti-nucleolin (C23) labeling revealed mature nucleoli in the most advanced rabbit SCNT (A) and bovine-rabbit (B) iSCNT embryos and their absence in porcine-rabbit (C) iSCNT embryos at 72 h after activation. To show nucleolin concentration in ring-like structures, the exposure time was increased from 2 s (A and B) to 16 s (C). Arrows showed nucleolin staining of fragmented chromatin (C) counterstained with DAPI to visualize DNA (D). Scale bar: 100 μ m.

Table 1 Interspecies somatic cell nuclear transfer (iSCNT) embryos: development and nucleoli formation.

Donor of nuclei	Donor of oocytes	Taxonomical relations	Embryo with nucleoli/embryo analyzed ^c	N	Cleaved %	Advanced embryos	
						D3 ^a N (% ^d)	N at the end of IVC ^b (Day, N of nuclei)
Cattle	Cattle	Intra-species	20/20	45	96	26 (58)	21 (D6, CM/BL)
Buffalo		Inter-genus	24/24				
Granulosa AF	Pig	Inter-subfamily	26/26	103	96	47 (46)	10 (D6, 20–27 nuclei)
Sheep			247	133	85	64 (48)	4 (D6, CM)
Mouse			0/30	62	98	43 (70)	37 (D4, 12–16 cells)
Cat			0/10	50	97	10 (20)	10 (D4, 10–16 cells)
Dog			0/31	50	97	37 (74)	37 (D4, 10–16 cells)
Rabbit			0/24	40	ND	24 (60)	24 (D4, 10–16 cells)
Horse			0/20	32	94	20 (63)	20 (D4, 12–16 cells)
Pig			0/35	423	94	230 (54)	230 (D6, 8–25 cells)
Pig			18/18	30	85	22 (73)	11 (D6, CM/BL)
Horse			21/43	79	81	41 (52)	8 (D6, 6–7 nuclei)
Rabbit	14/17	153	91	89 (58)	22 (D6, 5–10 nuclei)		
Dog	6/21	70	96	58 (83)	58 (D6, 4–6 cells)		
Cat	4/20	61	90	27 (44)	27 (D4, 4–6 cells)		
Mouse	0/18	31	ND	18 (58)	18 (D4, 4–6 cells)		
Sheep	6/26	34	94	28 (82)	28 (D4, 4–6 cells)		
Buffalo	0/22	30	ND	22 (73)	22 (D4, 4–6 cells)		
Cattle	0/31	249	84	131 (53)	131 (D7, 4–6 cells)		
Rabbit	Rabbit	Intra-species	26/26	96	83	44 (46)	21 (D3, CM/BL)
Cattle		Inter-order	10/44	70	90	23 (33)	23 (D3, 8–16 nuclei)
Pig		Inter-order	0/26	111	70	10 (9)	10 (D3, 8–9 nuclei)

AF, adult fibroblasts; CM, compacting morula; BL, blastocyst; ND, not determined.

^aAdvanced embryos D3: if cattle or rabbits are the oocyte donors – embryos with ≥ 8 blastomeres; if pigs are the oocyte donors – embryos with ≥ 4 blastomeres. ^bDevelopment was estimated at the end of embryo culture as the number of cells or nuclei stained with Hoechst. ^cBovine (≥ 8 cells) and porcine (≥ 4 cells) SCNT and iSCNT embryos were analyzed after 96 h of IVC. Rabbit SCNT and iSCNT embryos with ≥ 4 cells were analyzed after 72 h of IVC. ^dPercentage from total number of reconstructed embryos.

embryos, suggesting that active RNA polymerase I (AD test) guided rRNA synthesis. Moreover, some embryos were able to form compacting morula on day 6 with large active polygonal nucleoli. Donor cell culture effects on the preimplantation development of SCNT embryos might be even more profound in the case of iSCNT embryos and explains the difference in developmental competence of buffalo–bovine iSCNT embryos in our experiments and other laboratories (Kitiyant *et al.* 2001, Saikhun *et al.* 2002, Lu *et al.* 2005). Cattle–water buffalo hybrid IVF BLs serve as another confirmation of nuclear–cytoplasm compatibility between these species (Kochhar *et al.* 2002).

Hamilton *et al.* (2004) have shown that bovine–ovine iSCNT embryos were able to form ruminant-type NPBs as well as structures that appeared as fibrillar material surrounded by a rim of electron-dense granules, perhaps formerly of nucleolar origin. Taking all the above mentioned similarities of ovine and bovine embryos and the formation of numerous small nucleoli into account, it is possible to suppose some nuclear–cytoplasmic compatibility that guarantees at least partial EGA, i.e. RNA polymerase I function and rDNA transcription, in ovine–bovine iSCNT embryos. In contrast to Hua *et al.* (2008), who obtained almost 25% of BLs with 117 ± 13 cells/BL on day 6, we were not able to obtain morulae or BLs of such ovine–bovine

iSCNT embryos. The development of our iSCNT embryos, as well as that of hybrid embryos between bovine oocytes and ram sperm (Slavik *et al.* 1997), was blocked at the time of EGA. However, we observed similar morphology of bovine, ovine, and buffalo nuclei in bovine cytoplasm, which might indicate at least partial embryonic genome activity, in contrast to the complete block of embryo development at the stage of mitotic chromosome condensation in all iSCNT embryos that failed to form nucleoli.

In contrast to bovine oocytes, we found that porcine oocytes were able to support nucleoli formation in nuclei of many species: equine, rabbit, canine, and feline that possess very close EGA at the 4- to 8-cell stage (Grøndahl & Hyttel 1996, Hoffert *et al.* 1997, Kanka 2003, Maddox-Hyttel *et al.* 2005), and, interestingly, even in some ovine nuclei with EGA at the 8-cell stage (Crosby *et al.* 1988). The most intensively nucleolin-labeled nucleoli were found in equine and rabbit nuclei. We confirmed by AD test the existence of active RNA polymerase I transcription of rDNA in equine–porcine iSCNT embryos. However, none of these embryos with active RNA polymerase I was able to overcome the developmental block at the 4- to 8-cell stage, confirming the partial and aberrant character of EGA.

Using transmission electron microscopy, Hamilton *et al.* (2004) showed that the ovine ooplasm directs

initial nucleolar formation but is incompatible with the porcine nucleus for completing this event and forming fibrillar-granular nucleoli or restoring rRNA transcription (absence of ^3H -uridine incorporation). These authors demonstrated the absence of nucleolin-labeled nucleoli at 96 h in the most advanced 8- to 16-cell embryos. In our study, there was no doubt to the existence of nucleoli in ovine-porcine iSCNT embryos; however, the intensity of nucleolin staining and the thickness of shell-like nucleoli suggested incompleteness of nucleoli formation, or their immaturity, in comparison with nucleoli of control embryos or even in equine-porcine iSCNT ones.

Analyzing the embryo genome activation in bovine-porcine iSCNT embryos, we found no anti-nucleolin-stained nucleoli at any time point (48–96 h) studied either in bovine-porcine or in porcine-bovine iSCNT embryos. These embryos were characterized by prevailing nucleoplasmic nucleolin localization with occasional concentration around NPBs in some bovine and porcine nuclei. This pattern of nucleolin staining suggests the inability of these embryos to activate RNA polymerase I transcription and nucleoli formation and was identical to the pattern of nucleolin labeling in bovine and porcine SCNT embryos after inhibition of RNA polymerase I activity or EGA (Lagutina *et al.* 2010). A similar pattern of nucleolin staining was found in all studied iSCNT embryos that did not form nucleoli in our experiments and was also shown for porcine-ovine (Hamilton *et al.* 2004) and rhesus monkey-bovine (Song *et al.* 2009) iSCNT embryos. The block of EGA in bovine-porcine or porcine-bovine iSCNT embryos was confirmed by the absence of RNA polymerase II accumulation and its low activity, the absence of *Nanog* gene expression, a significantly lower level of mitochondrial mass in comparison with intra-species embryos, and a complete block of development at the 16- to 25-cell stage and 4-cell stage of iSCNT embryos, produced with bovine and porcine oocytes respectively (Lagutina *et al.* 2010). Our data contradict previous reports (Dominko *et al.* 1999, Uhm *et al.* 2007) of 4–8% BL rates, implying at least partial xeno-genome activation in a wide set of iSCNT embryos. Interestingly, the absence of formation of active mature nucleoli in rhesus monkey-bovine iSCNT embryos (Song *et al.* 2009) and their inability to pass the developmental block and form BLs were not associated with the complete absence of EGA-dependent nucleolar proteins, such as UBF and fibrillarin. The immunocytochemical staining revealed their presence in some monkey nuclei, which may be interpreted as a sign of low-level expression of these genes and potentially aberrant embryonic genome activity.

In respect to nucleus-cytoplasm incompatibility, the most unexpected finding was nucleoli formation by bovine nuclei but not by porcine nuclei in iSCNT embryos derived from rabbit oocytes. This observation

raises the question – what is the reason of ability/inability of oocyte cytoplasm to activate RNA polymerase I transcription? This might reflect the structural differences of NPBs and subsequent nucleoli formation between cattle and pig that represent two different types of nucleoli formation. The vacuolation of NPBs, penetration of rDNA and subsequent rRNA synthesis, and DFC formation in the body of NPB in nucleoli of ruminant type are completely different to that in porcine without vacuolation of NPBs, attachment of rDNA, and initiation of DFC formation in the periphery of NPBs. However, these structural differences do not explain the initiation of nucleoli formation in ovine-porcine iSCNT embryos (different types of nucleoli) and the absence of nucleoli in mouse-porcine iSCNT embryos (similar types of nucleoli (Flechon & Kopecny 1998, Maddox-Hyttel *et al.* 2007).

The simplest cause of nucleoli formation failure in iSCNT embryos may be a structural difference of promoter selectivity factors that play an important role in the formation of the RNA polymerase I complex on the promoter. These promoter selectivity factors were found to be species specific in human (SL1) and mouse (TIF-IB; Heix *et al.* 1997). These factors were not studied in other species. However, the ability of *Xenopus* rRNA to be transcribed in mouse cell extract (Culotta *et al.* 1987) supposes that species divergence is not so large, and nucleoli formation in donor nuclei of different unrelated species in rabbit and porcine cytoplasm also supports this.

It was found that ooplasm from different species have different demethylation capacity (Beaujean *et al.* 2004, Chen *et al.* 2004, 2006). The demethylation of repetitive sequences of the donor genome is determined by the recipient ooplasm and not by intrinsic properties of the donor nucleus (Chen *et al.* 2004, 2006). It seems that the high demethylation capacity of porcine oocytes can explain their ability to support initiation of nucleoli formation in nuclei of several species. In contrast, it was shown that rabbit oocytes possess much lower demethylation capacity than porcine oocytes (Shi *et al.* 2004). Together, with hypermethylation of porcine fibroblast nuclei (Chen *et al.* 2006), this may be the reason for failure of nucleoli formation in porcine-rabbit iSCNT embryos. However, rabbit oocytes were shown to support preimplantation development of embryos derived from nuclei of several species, including bovine (Jiang *et al.* 2006), Capra ibex (Jiang *et al.* 2005), chicken (Liu *et al.* 2004), camel and Tibetan antelope (Zhao *et al.* 2006), macaca (Yang *et al.* 2003), cat and panda (Wen *et al.* 2005), and even human (Shi *et al.* 2008). However, microarray analysis failed to detect significant human genome reprogramming in human-rabbit iSCNT embryos (Chung *et al.* 2009). There are different methylation patterns of early rabbit and bovine SCNT/IVF embryos with equally high methylation levels of the paternal and maternal genomes from the zygote up to the 16-cell stage in rabbit IVF

and SCNT embryos (Shi *et al.* 2004) in contrast to considerable demethylation in bovine embryos (Dean *et al.* 2001). The formation of nucleoli in bovine–rabbit iSCNT embryos in our experiments suggests that the level of bovine genome demethylation in rabbit cytoplasm was essential for at least partial bovine EGA.

The ability of oocyte to demethylate gene promoters has donor nucleus species-specific features (Wang *et al.* 2009). Although the trend of global demethylation seems to be similar in bovine SCNT and chimpanzee–bovine iSCNT embryos, bovine ooplasm could not recapitulate in chimpanzee nuclei the DNA demethylation events observed in the bovine SCNT embryos. These deficiencies could potentially significantly limit the transcription of chimpanzee-specific transcripts in the iSCNT embryos. This may be one of the reasons of nucleoli activation failure in bovine, buffalo, and mouse nuclei in porcine cytoplasm, as well as of nucleoli formation in bovine nuclei of bovine–rabbit iSCNT embryos.

We proposed that initiation of nucleoli formation depends on certain coordination of EGA in terms of cell stage. Analyzing the data, we found that nucleoli formation 1) always failed when EGA cell stage of donor nucleus was very different from EGA cell stage of recipient oocyte (mouse–bovine, mouse–porcine, and porcine–bovine iSCNT embryos), 2) was successful when EGA cell stages of donor and recipient species were similar, as seen with ovine, buffalo, and bovine nuclei in bovine cytoplasm or with porcine, rabbit, equine, feline, and canine nuclei in porcine cytoplasm. However, the similarity of EGA cell stage as the cause of nucleoli formation is not consistent as seen with porcine–rabbit (similar EGA cell stage but absence of nucleoli), or bovine–rabbit and ovine–porcine (different EGA cell stage but active nucleoli) iSCNT embryos.

Anti-nucleolin labeling provides us only limited information about the formation of nucleoli. As nucleolin is tightly connected with active RNA polymerase I, we can speculate about attachment of RNA polymerase I to rDNA promoter and initiation of transcription. Formation of transcriptionally active RNA polymerase I complex on rDNA promoter depends on the presence of RNA polymerase I, UBF, and several transcription factors. RNA polymerase I, UBF, fibrillarin, and other key nucleolar proteins are required for *de novo* embryonic transcription (Svarcova *et al.* 2007). The presence of UBF and fibrillarin in the NPB of rhesus monkey–bovine iSCNT embryos has been revealed by immunostaining (Song *et al.* 2009). Although these embryos failed to form active mature nucleoli, it can be concluded that bovine cytoplasm can guide some monkey genome activity. In our study, the formation of large nucleoli in the nuclei of different species in xeno-cytoplasm serves as a marker of partial EGA.

The efficiency of preimplantation SCNT embryo development (Boiani *et al.* 2005, Lagutina *et al.* 2006) depends on embryo culture conditions. The effect of IVC

medium on iSCNT embryo development (Yang *et al.* 2003, Liu *et al.* 2004, Zhao *et al.* 2006, Sugimura *et al.* 2009) is even more crucial, as it should support maternal RNA translation as well as EGA in xeno-ooplasm. It seems that medium efficiently supporting BL development of control SCNT embryos would sufficiently support the development of iSCNT embryos derived from the same oocytes (Zhao *et al.* 2006). However, there might be also nuclear donor-species preference of IVC medium in spite of the same oocyte source (Yang *et al.* 2003, Zhao *et al.* 2006). The data on the ability of IVC medium to support iSCNT embryo development are very contradictory. We also failed to reproduce the development of porcine–bovine iSCNT BLs in CR1 (Dominko *et al.* 1999, Uhm *et al.* 2007). SOF (synthetic oviduct fluid) *in vitro* embryo culture medium that is equally efficient for bovine and porcine IVF/NT pre-implantation development not only failed to support the development but also was not able to initiate EGA in porcine–bovine iSCNT embryos (Lagutina *et al.* 2010). We were not able to obtain ovine–bovine iSCNT BLs in SOF (Hua *et al.* 2008) despite nucleoli formation as a sign of EGA. Undoubtedly, there is still a wide field for investigation of *in vitro* maturation and embryo culture medium effects on the EGA in iSCNT embryos.

In conclusion, oocytes of different species have different abilities to activate nucleoli formation in iSCNT embryos. The time and cell stage of nucleoli formation in iSCNT embryos are under the control of recipient ooplasm. Formation of nucleoli, indicating at least partial EGA, is an essential but not entirely sufficient condition to overcome developmental block of iSCNT embryos at the time of transition from maternal to embryonic control.

Materials and Methods

Unless otherwise stated, all chemicals were purchased from Sigma–Aldrich.

Preparation of donor cells for nuclear transfer

Bovine, porcine, water buffalo, ovine, equine, rabbit, canine, feline, and mouse skin adult fibroblasts from passages 1 to 10 were used as donor cells for iSCNT into bovine and porcine oocytes. Cells were cultured in DMEM+TCM 199 (1:1) with 10% of FCS and 100 IU/ml penicillin and 100 µg/ml streptomycin in a humidified atmosphere of 5% CO₂, 5% O₂, and 90% N₂ at 38.5 °C. Two days before SCNT, the donor cells were passaged, and the day after, they were serum starved (0.5% FCS) for 24 h.

Bovine, rabbit, and porcine fetal fibroblasts from passages 2 to 6 were used as donor cells for iSCNT into rabbit oocytes. Cells were cultured in DMEM (Gibco) supplemented with 10% (v/v) FCS (Biochrom, Berlin, Germany), 2 mM L-glutamine, 0.1 mM 2-mercaptoethanol, 2 mM non-essential amino acids (Sigma), 100 IU/ml penicillin, and 100 µg/ml streptomycin in a humidified atmosphere of 5% CO₂ in air at 37 °C until confluence.

Bovine and porcine oocyte maturation

Oocytes were aspirated from 3 to 8 mm diameter ovarian follicles of slaughtered animals. *In vitro* manipulation was carried out essentially as described previously (Lagutina *et al.* 2006). Bovine oocytes were matured in TCM 199 supplemented with 10% (v/v) FCS, 1 µl/ml ITS Media Supplement (insulin, transferrin, and selenite, Sigma, cat. no. I1884), 1 mM sodium pyruvate, 0.5 mM L-cysteine, 10 mM glycine, 100 µM β-mercaptoethanol, and 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 cystine, 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 analog), 50 ng/ml Long epidermal growth factor (EGF, recombinant EGF analog), 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). For porcine IVF embryos, after 42 h of *in vitro* 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 epinephrine, 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 Ca²⁺-free TALP, and pelleted by centrifugation for 10 min at 400 g.

Nuclear transfer

SCNT 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. SCNT 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 (5 µg/ml) and Hoechst (5 µg/ml) for 5 min. Metaphase chromosomes were removed under u.v. light with a blunt enucleation pipette. Each cytoplasm was then 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. Cell couplets were washed in 0.3 M mannitol (Ca²⁺-free, 100 µM Mg²⁺) solution, fused by double DC pulses 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 (6-DMAP) 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 Ca²⁺ (Cheong *et al.* 2002) and 100 µM Mg²⁺, 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

SCNT and iSCNT embryos were cultured according to the Well of the Well method (Vajta *et al.* 2000) individually in microwells in 20 µl drops of m-SOFaa in 5% CO₂, 5% O₂, and 90% N₂ humidified atmosphere at 38.5 °C. Half the medium was renewed with fresh m-SOFaa on days 4 and 6 (day 0 was the day of SCNT). Cleavage was assessed 48 h after activation and the occurrence of BLs on days 6 and 7. All experiments comprised three or more replicates (unless otherwise indicated).

To assess the effect of inhibition of RNA polymerase I complex activity on the behavior of nucleoli, buffalo-bovine and equine-porcine iSCNT embryos were cultured in the medium supplemented with 0.2 µg/ml AD from 93 h until 96 h after activation.

Rabbit oocytes: nuclear transfer, fusion, activation, and embryo culture

Oocytes were obtained from sexually mature Zika breed rabbits. Female rabbits were superovulated by injection of 100 IU pregnant mares' serum (Sigma-Aldrich) i.m. and 100 IU human chorionic gonadotrophin (hCG; Sigma-Aldrich) i.v. 72 h later. Mature oocytes were flushed from the oviducts 15–16 h post-hCG injection in warm PBS supplemented with 4 mg/ml BSA. Cumulus cells were removed by gentle pipetting with a small-bore pipette after treatment of oocytes in 5 mg/ml hyaluronidase prepared in M199 (TCM 199 containing 10% FCS) for 15 min at 38.5 °C.

To induce the MII metaphase protrusion, cumulus-free oocytes were treated with 0.6 µg/ml demecolcine in M199 for 40 min to 2 h. The extruded MII metaphase with a small amount of associated cytoplasm was removed in M199 supplemented with 7.5 µg/ml cytochalasin B and 0.6 µg/ml demecolcine. Enucleated recipient oocytes were kept in M199.

An individual nuclear donor cell was introduced under the zona pellucida of an enucleated oocyte in M199.

Reconstructed embryos were produced after fusion of karyoplast–cytoplasm complexes in Eppendorf fusion medium (Hamburg, Germany) with an Eppendorf Multiporator (Eppendorf) using a double electric pulse of 1.95 kV/cm for 25 μ s. After 20–40 min incubation in M199, fused embryos were activated by the same electric pulse, then immediately incubated for 1 h in 1.9 mM 6-DMAP and 5 μ g/ml cytochalasin B prepared in Menezé B2 medium (INRA, Paris, France) containing 10% FCS in a humidified atmosphere of 5% CO₂ in air at 38.5 °C. After activation, embryos were cultured in Menezé B2 with 10% FCS medium for 72 h in a humidified atmosphere of 5% CO₂ in air at 38.5 °C.

Immunocytochemistry

IVF (after zona pellucida digestion with 0.5% pronase), SCNT, and iSCNT embryos at 48, 72, and 96 h after fertilization/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 polymerase I activity; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA, sc-8031) overnight. Embryos were washed in PBS/BSA and incubated with the secondary antibody conjugated with FITC (Santa Cruz Biotechnology, Inc.) at room temperature for 2 h, washed in PBS/BSA, and mounted in Cytifluor with DAPI.

Images

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

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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