Nuclear–cytoplasmic incompatibility and inefficient development of pig–mouse cytoplasmic hybrid embryos

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Abstract

Inter-species somatic cell nuclear transfer (iSCNT) embryos usually fail to develop to the blastocyst stage and beyond due to incomplete reprogramming of donor cell. We evaluated whether using a karyoplast that would require less extensive reprogramming such as an embryonic blastomere or the meiotic spindle from metaphase II oocytes would provide additional insight into the development of iSCNT embryos. Our results showed that karyoplasts of embryonic or oocyte origin are no different from somatic cells; all iSCNT embryos, irrespective of karyoplast origin, were arrested during early development. We hypothesized that nuclear–cytoplasmic incompatibility could be another reason for failure of embryonic development from iSCNT. We used pig–mouse cytoplasmic hybrids as a model to address nuclear–cytoplasmic incompatibility in iSCNT embryos. Fertilized murine zygotes were reconstructed by fusing with porcine cytoplasts of varying cytoplasmic volumes (1/10 (small) and 1/5 (large) total volume of mouse zygote). The presence of pig cytoplasm significantly reduced the development of mouse zygotes to the blastocyst stage compared with control embryos at 120 h post-human chorionic gonadotropin (41 vs 6 vs 94%, $P < 0.05$; 1/10, 1/5, control respectively). While mitochondrial DNA copy numbers remained relatively unchanged, expression of several important genes namely *Tfam*, *Polg*, *Polg2*, *Mfn2*, *Slc2a3* (*Glut3*), *Slc2a1* (*Glut1*), *Bcl2*, *Hspb1*, *Pou5f1* (*Oct4*), *Nanog*, *Cdx2*, *Gata3*, *Tcfap2c*, *mt-Cox1* and *mt-Cox2* was significantly reduced in cytoplasmic hybrids compared with control embryos. These results demonstrate that the presence of even a small amount of porcine cytoplasm is detrimental to murine embryo development and suggest that a range of factors are likely to contribute to the failure of inter-species nuclear transfer embryos.

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Introduction

Somatic cell nuclear transfer (SCNT) has immense potential in the conservation of endangered species and for the production of autologous human embryonic stem cells. However, in either case, the availability of oocytes from the same species is either limited due to biological constraints, as is the case for endangered species, or restricted due to other reasons including ethical and moral values. To overcome these difficulties, an alternative approach is to use oocytes from another species that are in plentiful supply, so-called inter-species SCNT (iSCNT). The first report of iSCNT used bovine oocytes as cytoplasm recipients for somatic cells of a range of species including sheep, pig, monkey, and rat

(Dominko *et al.* 1999). Since then, numerous reports have used a variety of combinations of recipient and donor species. Although there have been contrasting reports on the developmental competence of iSCNT embryos, it is evident that efficiency of iSCNT is extremely poor and inversely proportional to the evolutionary distance among the species (reviewed in Beyhan *et al.* (2007)). The majority of embryos produced by iSCNT, other than those from closely related species (Loi *et al.* 2001, Meirelles *et al.* 2001, Gomez *et al.* 2003, Li *et al.* 2006), failed to progress beyond preimplantation stages (Beyhan *et al.* 2007). In view of the contrasting reports and extremely poor developmental efficiency, development of iSCNT embryos, other than those from closely related species, to the blastocyst stage remain

elusive. Although, in humans, a single study reported isolation of an ES cell line from blastocyst stage embryos produced by iSCNT using rabbit oocytes (Chen *et al.* 2003b, Chung *et al.* 2009), similar rabbit–human iSCNT embryos produced in a recent study (Chung *et al.* 2009) all resulted in early embryonic arrest.

Among those factors that influence the outcome of SCNT, two factors that are particularly associated with oocyte cytoplasm are very likely to affect the outcome of iSCNT. These are incomplete or partial remodeling of the donor genome by heterogenous cytoplasm and nuclear–cytoplasmic incompatibility, including incompatibility between mitochondrial DNA (mtDNA) and nuclear DNA. We have addressed both of these issues using three experimental models. First, incomplete remodeling of the donor genome was addressed using donor nuclei from embryonic stages that may require less rigorous reprogramming than that from a somatic cell. Following SCNT, the somatic cell nuclei must undergo extensive reprogramming for successful development of cloned to embryos to blastocyst or beyond. However, it was recently demonstrated that the somatic cell nucleus undergoes only partial or incomplete reprogramming in iSCNT embryos (Chung *et al.* 2009, Wang *et al.* 2009). We hypothesized that using a donor genome that requires less extensive reprogramming than somatic cells may improve the frequency of development to the blastocyst stage or alternatively provide an improved model to study nuclear cytoplasmic interactions between different species. Therefore, in the first instance, we used embryonic blastomeres in place of somatic cells for iSCNT and compared pre-implantation embryo development with embryos produced by intra-species SCNT, iSCNT, and parthenogenetic activation. In the second series of experiments, reciprocal inter-species spindle transfer (iSPNT) was carried out between sheep and cow oocytes. Reprogramming of oocyte spindle by the oocyte on parthenogenetic activation is fairly easy to achieve and parthenogenetic ES cells have been established in both mice (Kaufman *et al.* 1983) and primates (Cibelli *et al.* 2002, Kim *et al.* 2007). Reprogramming of oocyte spindle again may not need an extensive reprogramming as somatic or embryonic nuclei.

Incompatibility between the nuclear and mitochondrial genome was reported to have compromised mitochondrial generation of ATP leading to embryonic arrest (Lanza *et al.* 2000, Loi *et al.* 2001, Trounce & Pinkert 2007, Bowles *et al.* 2008, Gomez *et al.* 2009). Xenoplasmic transfer (XOT) in somatic cell models is a valuable tool to address nuclear and mtDNA interactions between different species (Kenyon & Moraes 1997, Trounce & Pinkert 2007). Unlike iSCNT embryos that usually arrest around the stage of EGA, XOT embryos develop beyond EGA (Sansinena *et al.* 2011) and even into live offspring (Chiaratti *et al.* 2010). Thus the XOT approach would provide a better model to address nuclear and cytoplasmic cross talk between different species. Using this approach, we evaluated embryo development, mtDNA copy number, the expression profiles of a number of mitochondrial and pluripotent genes, and methylation of the *Pou5f1* promoter in pig–mouse cytoplasmic hybrid embryos.

Results

In vitro development of SCNT, iSCNT, SPNT, iSPNT, and parthenogenetic embryos

To investigate the development potential of inter-species embryos of different taxonomic distance in evolution, we generated inter-order (mouse–pig), inter-suborder (porcine–ovine), and inter-subfamily (ovine–bovine) embryos by somatic or embryonic cell nuclear transfer. However, none of the embryos from any of the above combinations developed to blastocyst stage. All the above iSCNT embryos arrested at the stage of EGA specific to recipient cytoplasm. We anticipated that mouse–pig inter-order embryonic cloned embryos might be able to develop beyond the stage of EGA. However, they behaved no different from mouse–pig inter-order somatic cell cloned embryos. In controls, intra-species bovine (23.5%), ovine (17.3%), and porcine (17.8%) SCNT embryos developed to the blastocyst stage. No significant difference was observed in the proportion of embryos developing to blastocysts between intra- or inter-species SCNT groups (Table 1).

Table 1 Development of intra- and inter-species nuclear transferred reconstructed embryos.

	Donor cell	Cytoplasm	Total	No. of two cell (%)	No. of four cell (%)	No. of 8–16 cell (%)	No. of blastocyst (%)
Inter-order	Mouse cumulus	Porcine	110	79 (71.8) ^a	57 (51.8) ^a	0 (0.0) ^b	0 (0.0) ^c
	Mouse blastomere	Porcine	79	45 (57.0) ^b	36 (45.6) ^a	0 (0.0) ^b	0 (0.0) ^c
Inter-suborder	Porcine	Ovine	72	57 (79.2) ^a	NE	51 (70.8) ^a	0 (0.0) ^c
Inter-subfamily	Ovine	Bovine	79	67 (84.8) ^a	NE	61 (70.8) ^a	0 (0.0) ^c
Intra-species	Bovine	Bovine	68	59 (86.8) ^a	NE	48 (70.6) ^a	16 (23.5) ^a
	Ovine	Ovine	75	60 (80.0) ^a	NE	53 (70.7) ^a	13 (17.3) ^a
	Porcine	Porcine	101	85 (84.2) ^a	61 (60.4) ^a	NE	18 (17.8) ^a
Porcine parthenotes		Porcine	71	58 (81.7) ^a	54 (76.0) ^b	NE	40 (56.3) ^b

NE, not examined. ^{a,b,c} Different superscripts within the same column indicate significant difference ($P < 0.05$).

Table 2 Development of intra- and inter-species spindle transfer embryos.

Donor	Recipient	Total	No. of two cell (%)	No. of 8–16 cell (%)	No. of blastocyst (%)
Ovine spindle	Ovine oocyte	49	39 (79.6) ^a	35 (71.4)	9 (18.4) ^a
Ovine spindle	Bovine oocyte	46	41 (89.1) ^{a,c}	35 (79.1)	0 (0.0) ^b
Bovine spindle	Bovine oocyte	52	42 (80.8) ^a	38 (73.0)	15 (28.6) ^a
Bovine spindle	Ovine oocyte	58	55 (94.83) ^{b,c}	47 (81.0)	0 (0.0) ^b

^{a,b,c} Different superscripts within the same column indicate significant difference ($P < 0.05$).

To eliminate effects of somatic cell reprogramming process on cytoplasmic compatibility between two different species, we produced inter-subfamily embryos (ovine–bovine and bovine–ovine) by reciprocal exchange of spindles between MII cow and sheep oocytes (iSPNT). Intra-species spindle transfer (SPNT) embryos produced by fusing MII spindles to enucleated oocytes of the same species were used as controls. Bovine and ovine SPNT embryos developed beyond EGA stages (71.4 and 73.1%, respectively; $P > 0.05$) and developed to the blastocyst stage (18.4 and 28.9%, respectively; $P > 0.05$) at day 7. However, neither cow/sheep nor sheep/cow iSPNT embryos developed beyond the 8- to 16-cell stage, although the proportion that developed to 8- to 16-cell stages was not significantly different from SPNT controls ($P > 0.05$; Table 2).

Development of pig–mouse cytoplasmic hybrid embryos

Pig–mouse cytoplasmic hybrids were produced by fusing cytoplasts containing small volume cytoplasm hybrid (SVCH) or large volume cytoplasm hybrid (LVCH) volumes of pig cytoplasm to mouse zygotes. Mouse zygotes manipulated to remove the second polar body but not fused with pig cytoplasm and non-manipulated intact mouse zygotes were used as controls. The developmental potential of cytoplasmic hybrids to the two-cell stage or to morula/blastocyst stages was not different from control embryos. However, both LVCH and SVCH embryos were slow in their speed of development compared with control group of embryos from four-cell stage onward. While majority of control embryos became blastocysts, only a small fraction of SVCH and LVCH embryos became blastocysts at 96 h post-human chorionic gonadotropin (hCG). Nonetheless, the rate of cleavage and development to morula/blastocyst stage at 96 h post-hCG was not different

between SVCH and LVCH embryos. However, culture of cytoplasmic hybrids for a further 24 h resulted in a significant difference in the survival of cytoplasmic hybrids ($P < 0.01$). At 120 h post-hCG, while 41% of SVCH zygotes developed to the expanded blastocyst stage, almost 95% of LVCH had degenerated and only 5.6% of LVCH zygotes developed to the expanded blastocyst stage. In control group, at the same time point, 91.9 and 94.2% of manipulated and non-manipulated mouse zygotes had developed to the expanded blastocyst stage (Table 3; Fig. 1A, B and C).

To determine whether cytoplasmic hybrid embryos support post-implantation development, morula and blastocyst stage embryos from SVCH or control manipulated embryos at 96 h post-hCG were transferred to the uteri of 2.5-day pseudo-pregnant CD1 female mice. Although 20 of 53 embryos transferred in control group developed to live normal offspring (37.7%), no offspring were obtained from 177 embryos transferred in the SVCH group.

mtDNA fate in cytoplasmic hybrid embryos

In order to evaluate the fate of mtDNA in cytoplasmic hybrid embryos, LVCH embryos at the zygote (24 h post-hCG), two-cell (48 h), and morula/blastocyst stages (96 h) were collected and the mtDNA copy number per embryo determined by quantitative real-time PCR (qRT-PCR) and compared with respective stages of control (manipulated but not fused with pig cytoplasm) embryos. Both mouse and porcine mtDNA were detected in all stages. Mouse mtDNA copy numbers at one-cell zygote stage ($2\,56\,250 \pm 56\,195$ vs $2\,49\,800 \pm 46\,560$), two-cell stage ($1\,74\,791 \pm 27\,391$ vs $2\,13\,600 \pm 40\,420$), and morula/blastocyst stage ($1\,95\,166 \pm 54\,623$ vs $1\,58\,100 \pm 38\,270$) were not significantly different between cytoplasmic hybrid and control mouse embryos respectively ($P > 0.05$).

Table 3 Development of the pig–mouse cytoplasm hybrid embryos.

	No. of zygotes	No. of two cell at 44 h post-hCG (%)	No. of morula/blastocyst at 96 h post-hCG (%)	No. of blastocyst at 120 h post-hCG (%)
Small volume cytoplasm hybrid	190	190 (100)	186 (97.9)	78 (41.0) ^a
Large volume cytoplasm hybrid	107	103 (96.2)	100 (93.4)	6 (5.6) ^b
Manipulation (polar body removed) control	149	147 (98.6)	138 (92.6)	137 (91.9) ^c
Non-manipulated control	122	119 (97.5)	115 (94.2)	115 (94.2) ^c

^{a,b,c} Different superscripts within the same column indicate significant difference ($P < 0.01$).

Figure 1 (A) Development of the large volume pig-mouse cytoplasm hybrid embryos. a) mouse zygotes before fusion with pig cytoplasm; b) mouse zygotes after fusion with pig cytoplasm; c) two-cell; d) four-to-eight-cell; e) morula/blastocyst; f) expanded blastocyst. (B) Development of the small volume pig-mouse cytoplasm hybrid embryos. a) mouse zygotes before fusion with pig cytoplasm; b) mouse zygotes after fusion with pig cytoplasm; c) two-cell; d) four-to-eight-cell; e) morula/blastocyst; f) expanded blastocyst. (C) Development of the control mouse embryos. a) mouse zygotes manipulated to remove second polar body; b) two-cell; c) four-to-eight-cell; d) morula/blastocyst; e) expanded blastocyst. Pictures of two-cell, four-to-eight-cell, morula/blastocyst, and blastocyst were taken at 44, 66, 96, and 120 h post-hCG respectively (scale bar represents 50 μ m).

Mouse mtDNA numbers at different stages of development did not significantly differ and remained relatively constant in cytoplasmic hybrid or control embryos. The porcine mtDNA copy numbers in cytoplasmic hybrids remained constant ($P > 0.05$) throughout development from zygote ($11\,992 \pm 1211$) to two-cell (8912 ± 2524) and morula/blastocyst (7383 ± 769) stages (Fig. 2).

Aberrant expression of mitochondrial related genes in cytoplasm hybrid embryos

To determine whether the fate of mtDNA in the hybrid embryos was associated with the expression of genes involved in mtDNA replication, we examined nuclear-encoded factors such as mitochondrial transcription factors A (*Tfam*), mitochondrial-specific DNA polymerase gamma catalytic subunit (*Polg*) and

Figure 2 The number of mtDNA copies/embryo in cytoplasmic hybrid and control mouse embryos at different stages of pre-implantation development. mtDNA copy numbers were analyzed by real-time PCR. Mouse or pig mtDNA numbers remained constant across different developmental stages in both cytoplasm hybrid and control group of embryos ($P > 0.05$). Error bars represent the value (mean $\pm$ S.D.).

accessory (*Polg2*), and mitochondrial nuclear-encoded cytochrome *c* oxidase 1 (*mt-Cox1*) and *mt-Cox2*. Transcripts of all the examined genes were detectable but significantly downregulated in the cytoplasmic hybrid embryos compared with those in control mouse embryos (Fig. 3). In particular, expression of *Polg* and *Polg2* was significantly decreased to 5 and 7%, respectively, of the levels observed in controls ($P < 0.001$).

Aberrant expression of genes involved in differentiation and embryo viability

The majority of cytoplasm hybrid embryos failed to develop at the stage of morula–blastocyst transition that coincides with establishment of inner cell mass (ICM) and trophoblast (TE) lineages. Therefore we speculated that the genes involved in the establishment of these lineages were abnormally expressed in hybrid embryos. To test our hypothesis, we examined the

Figure 3 Gene expression profile of pig–mouse large volume cytoplasmic hybrid embryos. Expression of the genes involved in cell lineage establishment (TE and ICM), cell viability, and mtDNA replication in LVCH morula/blastocyst (96 h post-hCG) were compared with the control mouse embryos. Bars represent the value (mean $\pm$ S.D.). *No difference was observed from control mouse embryos ($P > 0.05$).

expression profiles of several transcription factors that play key roles in the establishment of TE and ICM lineage; caudal-related homeobox 2 (*Cdx2*), GATA binding protein 3 (*Gata3*), TEA domain family member 4 (*Tead4*), transcription factor AP-2, gamma (*Tcfap2c*), POU domain, class 5, transcription factor 1 (*Pou5f1*), and Nanog homeobox (*Nanog*) in LVCH embryos at 96 h post-hCG. Because most of the LVCH embryos had degenerated by 120 h post-hCG, we also evaluated several genes that are known to play a role in apoptosis: B-cell CLL/lymphoma 2 (*Bcl2*), heat shock 27 kDa protein 1 (*Hspb1*), mitofusin 2 (*Mfn2*), solute carrier family 2 (*Slc2a1*; also known as glucose transporter 1 (*Glut1*)), and *Slc2a3* (also known as *Glut3*). As shown in Fig. 3, except for *Tead4* ($P > 0.05$), transcripts of all the examined genes were significantly downregulated in cytoplasmic hybrids ($P < 0.05$). Among the above genes, expression of *Cdx2*, *Gata3*, *Slc2a1*, and *Slc2a3* was even more remarkably decreased ($P < 0.005$). The expression level of *Pou5f1*, however, was relatively higher than *Cdx2* and *Gata3* ($P < 0.001$) in hybrid embryos.

Reprogramming of pig–mouse cytoplasmic hybrid embryos

We also compared methylation patterns of a differentially methylated region in the promoter region of *Pou5f1*, a gene critical to the maintenance of pluripotency, and long interspersed nuclear element 1 (*Line1*), the most abundant repetitive sequence in mouse embryos between LVCH and control embryos at 96 h post-hCG. Methylation profiles of 11 conserved CpG dinucleotide sequence sites in *Pou5f1* and 12 in *Line1* were examined by bisulfite sequencing (Fig. 4). For *Pou5f1*, in control mouse embryos, all of these sites were unmethylated although 13.6% of CpG sites in the hybrid embryos were methylated ($P < 0.05$). The methylation status of 12 conserved CpG sites in *Line1* was not different between the two groups (both 43.3%).

Discussion

None of the inter-species nuclear transfer embryos in our study developed beyond the stage of EGA specific to the recipient oocyte. However, all intra-species SCNT embryos produced under the same conditions used for iSCNT developed to blastocyst stage. Our results were not different from several other recent reports (Chung *et al.* 2009, Wang *et al.* 2009, Lagutina *et al.* 2010), who had also reported that iSCNT embryos failed to reactivate the embryonic genome and arrested at the stage of EGA specific to the recipient oocyte. Conversely, inter-genus (Oh *et al.* 2006, Yin *et al.* 2006), inter-family (Dominko *et al.* 1999, Zhao *et al.* 2007), inter-order (Yang *et al.* 2003, Wen *et al.* 2005, Illmensee *et al.* 2006), and even inter-class iSCNT (Chen *et al.* 2002, Liu *et al.* 2004) nuclear transfer embryos were reported to have

Figure 4 DNA methylation profiles of the 5'-upstream region of the *Pou5f1* gene and repetitive sequence, *Line1*. Methylation profiles of conserved CpG sites in the *Pou5f1* and *Line1* were analyzed by bisulfite sequencing. Open circles represent unmethylated CpG sites and black closed circles represent methylated CpG sites. (B and D) LVCH embryos. (A and C) Control mouse embryos.

developed to the blastocyst stage. However, these later reports also observed that the majority of iSCNT embryos arrested around the stage of EGA and only a very small percent of embryos developed to blastocyst stage. Unfortunately, the origin of the blastocysts produced in the majority of these studies was not critically verified and except for those derived from closely related species failed to develop further (Beyhan *et al.* 2007). In view of the contrasting reports and the very poor efficiency of iSCNT, the developmental competence of iSCNT embryos other than closely related species beyond the stage of EGA is elusive. The overall efficiency of SCNT is often inefficient across species and stochastic (Campbell *et al.* 2007) and perhaps more pronounced in iSCNT embryos.

The efficiency of nuclear reprogramming in SCNT depends on the epigenetic signature/differentiated state of the donor nuclei (Campbell *et al.* 2007). Compared to somatic cells, embryonic nuclei from pre-implantation embryos are far less differentiated and the overall efficiency of embryonic cloning is higher than that when using somatic cells. Therefore, we hypothesized that choosing a donor cell that would require less extensive reprogramming than a somatic cell may provide some additional insight into the developmental potential of iSCNT embryos. Therefore at first, we have evaluated the development competence of mouse-pig inter-order embryonic cloned embryos and determined whether they would be able to progress beyond the four-cell stage, the stage at which EGA occurs in pig. However, these embryos were very similar to inter-order mouse-pig somatic cell cloned embryos in their development and also failed to progress beyond the

four-cell stage. As the media and incubation conditions required for culture differ between pig and mouse embryos, it can be argued that differences in the same may have limited inter-order mouse-pig embryonic development beyond four-cell stage. However, pig zygotic medium used for the culture of mouse-pig iSCNT embryos can support mouse embryonic development and mouse embryos can tolerate 39 °C, the temperature at which pig embryos are usually cultured (D Amarnath, I Choi, T Wakayama & KHS Campbell, unpublished observations). Therefore we can exclude that differences in culture conditions may have contributed to developmental arrest of mouse-pig inter-order embryos.

Then, we evaluated whether reducing evolutionary distance between the species used for iSCNT could improve development competence of iSCNT embryos. We have chosen bovine and ovine species as these species belong to the same family. Unlike mouse and pig embryos, bovine and ovine embryos are very much similar to each other in their *in vitro* maturation and culture conditions, kinetics of embryo development, and timing of EGA. We chose the spindle of MII oocytes as karyoplasts for reciprocal spindle transfer between sheep and cow oocytes. In contrast to somatic or embryonic nuclei, which are not designed for reprogramming by the oocyte, MII spindle is a natural component of oocytes and is designed for reprogramming by the oocyte. Both cow and sheep oocytes can be activated by the same parthenogenetic activation protocol and parthenogenetic development could be easily achieved with cow and sheep oocytes. Indeed, by choosing sheep and bovine oocytes we excluded many technical factors that may affect development. Control SPNT cow and sheep embryos as expected developed to the blastocyst stage; however, none of the cow-sheep or sheep-cow iSPNT embryos developed beyond the 8- to 16-cell stage, again the stage at which EGA occurs in both species. Taken together, our results support the argument that development of inter-species nuclear transfer embryos is highly inefficient with developmental arrest occurring at the stage of EGA specific to recipient cytoplasmic species (Chung *et al.* 2009, Wang *et al.* 2009, Lagutina *et al.* 2010). These recent reports have clearly demonstrated that the donor nucleus in iSCNT is only partially remodeled by the heterologous cytoplasm, and iSCNT embryos need to overcome nuclear-cytoplasmic compatibility issues before any further embryonic development to the blastocyst or later stages can be expected (Chung *et al.* 2009, Wang *et al.* 2009, Lagutina *et al.* 2010).

To delineate the effects of nuclear cytoplasmic incompatibility from the effects of inefficient nuclear reprogramming on development of iSCNT embryos, we used pig-mouse cytoplasmic hybrids in place of iSCNT. Independent of the volume of pig cytoplasm fused, both LVCH and SVCH embryos developed to the morula stage; however, development beyond the morula stage

was significantly influenced by the volume of pig cytoplasm fused to mouse zygotes. While 41% of SVCH embryos developed to blastocysts, only 6% of LVCH embryos developed from the morula to the blastocyst stage and the rest degenerated at 120 h post-hCG. Embryo transfer of SVCH morulae/blastocysts at 96 h post-hCG failed to develop into offspring. Although our results were different from a recent study (Chiaratti *et al.* 2010) that reported generation of homoplasmic bovine offspring from buffalo–cattle cytoplasm hybrids, the species involved in the later study belong to the same family and a hybrid was reportedly born from hybridization of buffalo and cattle in China and USSR (Mason 1976). Then, we investigated whether mitochondrial heteroplasmy contributed to developmental failure in cytoplasm hybrids. However, RT-PCR analysis of mtDNA of both pig and mouse in LVCH showed that the extent of heteroplasmy was minimal (Fig. 2). The percentage of pig mtDNA present in LVCH embryos was <5% of total mitochondrial numbers. In view of the presence of more than 95% mouse mtDNA of the total mtDNA in pig–mouse cytoplasm hybrid embryos and the birth of homoplasmic cattle from buffalo–bovine cytoplasm hybrids (Chiaratti *et al.* 2010), we speculate that mitochondrial heteroplasmy may not be a major cause of developmental failure in cytoplasmic hybrid embryos.

The fact that cytoplasmic hybrid embryos developed to four-cell and later stages indicate that these embryos have successfully undergone first and second wave of EGA at late one- and two-cell stages respectively. It was earlier demonstrated that EGA occurs in several waves and each wave peaks at different stages of mouse embryonic development and each wave should occur at appropriate stage of development for further development of embryos but not before or after (Hamatani *et al.* 2004). These authors suggested that mid pre-implantation gene activation (MGA), the third wave of genes that peak at the eight-cell stage are translated and function by the morula stage, supporting development to the blastocyst. We speculated that inefficient MGA at eight-cell and later stages in cytoplasmic hybrids may have been responsible for delaying or causing failure of the morula to blastocyst transition in SVCH and LVCH respectively. We examined the expression profile of several genes that are activated at eight-cell through to the stage of morula and blastocyst (Hamatani *et al.* 2004, Thundathil *et al.* 2005). Gene expression analysis showed that nuclear- and mitochondrial-encoded gene transcripts (*Tfam*, *Polg* and *Polg2*, *mt-Cox1* and *mt-Cox2*) were significantly downregulated in cytoplasmic hybrids. Although mtDNA replication does not occur until at least blastocyst stage (Thundathil *et al.* 2005), nuclear-encoded mitochondrial transcription and replication factors are significantly increased (Piko & Taylor 1987, Thundathil *et al.* 2005) and mitochondria undergo significant morphological changes from simple spherical mitochondria observed in MII oocytes to

become elongated with numerous cristae from eight-cell to blastocyst stages (Piko & Matsumoto 1976). It was suggested that such an increase in abundance of mitochondrial gene transcripts and mitochondrial morphological changes are required for an increased level of mitochondrial metabolism in morula and blastocyst stage embryos (Thundathil *et al.* 2005). Several metabolic activities such as oxygen consumption (Trimarchi *et al.* 2000), utilization of glucose (Leese & Barton 1984), ATP production (Sturmey & Leese 2003), and mitochondrial membrane potential (Acton *et al.* 2004) have been reported to increase at morula and blastocyst stages. It is possible that downregulation of mitochondrial gene transcripts in cytoplasmic hybrids at the morula stage may have compromised the efficiency of mitochondrial mediated metabolic activities and adversely affected the morula–blastocyst transition in cytoplasmic hybrids. Previous studies have shown that incompatibility between nuclear and mitochondrial genomes compromised mitochondrial generation of ATP in somatic cell cybrids (Kenyon & Moraes 1997, McKenzie & Trounce 2000, McKenzie *et al.* 2003, Trounce & Pinkert 2007) and iSCNT embryos (Chung *et al.* 2009, Wang *et al.* 2009, Lagutina *et al.* 2010) and could lead to the embryonic arrest (Wang *et al.* 2009).

Mitochondrial morphology and mtDNA copy number depend on the balance of fusion and fission activities. *Mfn1* and *Mfn2* play major roles in mitochondrial fusion. Mouse embryonic fibroblasts deficient in *Mfn1* and *Mfn2* displayed a severe reduction in mitochondrial fusion and fragmentation of mitochondrial tubules (Chen *et al.* 2003a). Perturbation of mitochondrial fusion results in defects in mitochondrial membrane potential and respiration, poor cell growth, and increased susceptibility to apoptosis (Chen & Chan 2005). Cytochrome *c* released from fragmented mitochondrial tubules binds to apoptotic protease activating factor 1 (APAF1) in the cytosol and induces apoptosis by activation of the caspase cascade (Bao & Shi 2007). It is conceivable that decreased expression of *Mfn2* in cytoplasmic hybrids may have caused a decrease in mitochondrial fusion, abnormal mitochondrial morphology, fragmentation of mitochondria, and an increased incidence of apoptosis. Furthermore, it is also possible that the fusion of a large volume of pig cytoplasm to mouse zygotes in LVCH embryos may have predisposed the embryos to disproportionate segregation of mitochondria between dividing blastomeres. Aberrations in normal pattern of mitochondrial translocation and distribution during early cleavage have implications on ATP generation in individual blastomeres and blastomeres with reduced inheritance of mitochondria failed to divide and eventually causing cell death in mouse embryos (Van Blerkom *et al.* 2000).

Glucose transporter genes were aberrantly expressed in cytoplasmic hybrid embryos. *Slc2a3* in association

with *Slc2a1* is responsible for uptake of majority of glucose from external milieu and downregulation of *Slc2a3* caused a significant reduction in glucose uptake in the mouse preimplantation embryos (Pantaleon *et al.* 1997). The expression of *Slc2a3* is essential for formation of blastocyst in mouse and this requirement is independent of its role in the transport of glucose (Pantaleon *et al.* 1997, Pantaleon & Kaye 1998). The switch to metabolize glucose as the energy source correlates with compaction of morula and is coincident with expression of *Slc2a3* (Pantaleon *et al.* 1997). *Slc2a1* signals external glucose concentration and enables the switch from pyruvate to glucose that coincides with formation of blastocyst (Pantaleon & Kaye 1998). The significant downregulation of both *Slc2a3* and *Slc2a1* in cytoplasmic hybrid morulae should have adversely affected glucose uptake, embryonic metabolism, blastocyst formation, and embryonic viability. Decreased glucose transporter expression and glucose transport triggers apoptosis in mouse preimplantation embryos (Moley *et al.* 1998, Chi *et al.* 2000). Apoptosis is regulated by the activity of pro- and anti-apoptotic genes during preimplantation development (Jurisicova *et al.* 1998). Therefore, we also evaluated the expression of anti-apoptotic genes such as *Bcl2* and *Hspb1* (Jurisicova *et al.* 1998, Exley *et al.* 1999, Choi *et al.* 2010); both the genes were significantly downregulated in cytoplasmic hybrids. Taken together, while the low abundance of *Mfn2*, glucose transporter genes increased the sensitivity of cytoplasm hybrids to apoptotic stimuli, poor expression of anti-apoptotic genes may have failed to prevent apoptosis. Although apoptosis maintains cellular quality in developing embryos by removing damaged cells, extensive apoptosis may lead to early embryonic death (Brill *et al.* 1999).

As the embryonic development of cytoplasmic hybrids is affected at the morula–blastocyst transition, we hypothesized that regulatory network of genes responsible for establishment and maintenance of the TE and the ICM may have also been affected. At this stage, *Tead4* activates the expression of the transcription factors *Cdx2* and *Gata3* that are required for the establishment of a functional TE (Yagi *et al.* 2007, Nishioka *et al.* 2008, Home *et al.* 2009, Ralston *et al.* 2010). *Cdx2* activates *Gata3* and transcription factor *Tcfap2c* (and vice versa), which in turn represses the pluripotency genes, *Nanog* and/or *Pou5f1* expression, leading to lineage restricted gene expression profiles (Niwa *et al.* 2005, Home *et al.* 2009, Kuckenberg *et al.* 2010). Poor expression of all the above genes in cytoplasmic hybrids may have adversely affected ICM and TE lineage segregation. The ion and fluid transport functions of the TE mediates the fluid dynamics that control cavitation and blastocyst formation (Watson & Barcroft 2001). The expression of *Cdx2* is restricted to TE and is required for TE formation (Yamanaka *et al.* 2006). The significant downregulation of *Cdx2* expression in cytoplasmic hybrids could have adversely affected TE formation and its fluid transport

functions leading to failure of blastocyst formation. Although it is not clear from our current experiments whether abnormal gene expression in LVCH embryos is a cause or consequence of developmental arrest due to failure of morula–blastocyst transition in LVCH embryos, it is obvious from our findings that abnormal gene expression in LVCH embryos is indeed associated with the presence of pig cytoplasm in mouse zygotes. Further studies involving gene expression analysis of embryos at different stages of development from two-cell onward may provide conclusive evidence on the above. We would also like to point out that inefficient embryonic development or abnormal gene expression in cytoplasmic hybrid embryos may not be a consequence of procedures used for fusion of pig cytoplasm to mouse zygotes. We (Van Thuan *et al.* 2006) and others (Cheng *et al.* 2009) have earlier shown that mouse–mouse ooplasm transfer does not have any adverse influence on development potential, gene expression, and DNA methylation of resulting embryos.

We further examined whether the aberrant gene expression profile in cytoplasmic hybrids is caused by changes in DNA methylation. We compared the methylation profile of CpG sites in the promoter region of the pluripotent gene *Pou5f1* and *Line1*, the most repetitive sequences between cytoplasm hybrid and control mouse embryos. While methylation of *Pou5f1* was different between the two groups, no difference was observed in *Line1*. Methylation at *Line1* regions is used as a proxy for measuring global methylation levels (Ostertag & Kazazian 2001, Chalitchagorn *et al.* 2004); implying global methylation levels were not different between the two groups. However, the mechanism by which the presence of pig cytoplasm affected gene expression of mouse embryos requires further investigation. Factors that could have potentially affected gene expression include RNA binding proteins, micro-RNAs, and maternal transcripts of pig origin in the cytoplasmic hybrids. RNA binding proteins play significant roles in mRNA editing, localization, stability, and translation (Yu *et al.* 2001, Saunders & Barber 2003) and microRNAs regulate post-transcriptional events (Blakaj & Lin 2008) in pre-implantation embryos. Timely translation of maternal transcripts is essential for optimal gene expression patterns during maternal to embryonic transition (Oh *et al.* 2000); however, failure of timely degradation of maternal transcripts is detrimental to further embryonic development (Alizadeh *et al.* 2005). While an intrinsic mechanism(s) should have regulated timely translation/degradation of maternal transcripts of mouse origin, whether such mechanism(s) are specific to mouse transcripts alone or equally effective against maternal transcripts of the pig in the cytoplasmic hybrid embryos is not known. Further studies are necessary to evaluate whether maternal transcripts are efficiently regulated in inter-species cloned (foreign chromatin) /cytoplasm hybrid (foreign cytoplasm)

embryos. Further studies are also needed to investigate whether differential half-life of maternal transcripts of mouse (late two-cell stage) and pig (four-cell stage) origin may have interfered with regulated degradation or translation of maternal transcripts in cytoplasm hybrid embryos.

In conclusion, we suggest that inter-species nuclear transfer embryos from distant species independent of the type of donor nucleus usually fail to develop beyond the stage of embryonic genomic activation that is specific to the recipient oocyte species. The presence of foreign ooplasm in a developing embryo of another species is detrimental to embryonic development. The stage at which embryonic development is affected in cytoplasmic hybrid embryos is dependent on the amount of foreign cytoplasm and the species involved. Taken together, our results show that while iSCNT embryos (100% foreign ooplasm) arrest around the stage of embryonic genomic activation, inter-species cytoplasmic hybrid embryos containing small or moderate amounts of foreign ooplasm undergo developmental failures at later stages of pre- or post-implantation development, depending on the amount of foreign ooplasm. From our results, we argue that inter-species nuclear transfer embryos that contain cytoplasm from one species and karyoplast from another distant species/class/order need to overcome a number of nuclear–cytoplasmic incompatibilities before development to blastocyst or the production of live offspring can be expected.

Materials and Methods

All chemicals and reagents were purchased from Sigma–Aldrich otherwise stated.

Preparation of oocytes

Bovine, ovine, and porcine *in vitro* maturation was performed as described previously (Lagutina *et al.* 2007, Choi & Campbell 2010, Kelly *et al.* 2010). In brief, cumulus–oocyte complexes (COC) with uniform cytoplasm and several layers of cumulus cells were selected and then incubated in species specific *in vitro* maturation media under mineral oil at 39 °C in a humidified atmosphere of 5% CO₂ in air for 24 (bovine and ovine) and 42 h (porcine). Groups of ~40 bovine or ovine COC were cultured in bicarbonate-buffered TCM199 with Earle's salts (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 5 µg/ml FSH (NIDDK, NIH, Bethesda, MD, USA) and LH, 1 µg/ml 17β-estradiol, and 50 µg/ml gentamycin, and additionally 100 µM cysteamine and 0.3 mM sodium pyruvate for ovine. For porcine *in vitro* maturation, bicarbonate-buffered TCM199 with Earle's salts was supplemented with 10% FBS 0.3 mM L-glutamine, 0.57 mM cysteine, 10 mg/ml EGF, 0.5 µg/ml LH and FSH, 50 µg/ml for the first 22 h, and then changed to fresh medium without hormones for the remainder of the maturation period.

Nuclear transfer

Nuclear transfer was performed using a zona-free method as described (Obach *et al.* 2003, Lagutina *et al.* 2007). In brief, *in vitro* matured oocytes were denuded of cumulus cells by gentle vortexing and /or pipetting after brief exposure to hyaluronidase (300 IU/ml), and oocytes with a visible first polar body were selected for nuclear/spindle transfer or parthenogenetic activation. The zona pellucida was removed from the oocytes by incubating in 5 mg/ml pronase for 2–3 min. Prior to enucleation, porcine oocytes were exposed to Hoechst 33342 (5 µg/ml) and cytochalasin B (CB; 7.5 µg/ml) for 5 min 39 °C. The position of the metaphase spindle within the oocyte was localized by a brief exposure to u.v. light, enucleation was then performed in the presence of CB using a blunt pipette. Bovine and ovine zona-free MII oocytes were incubated in demecolcine (0.5 µg/ml) for 30 min to induce protrusion of the metaphase spindle. Enucleation was carried as described previously (Amarnath *et al.* 2007) by removing the membrane protrusion containing metaphase spindle using a blunt micropipette. Enucleated oocytes were individually washed for few seconds in phytohemagglutinin P (300 µg/ml) and then quickly dropped and rolled over a single donor cell. For intra-species SCNT in cattle, sheep, and pig, a quiescent primary fetal fibroblast was used as the nuclear donor cell. For mouse–pig iSCNT using an embryonic donor nucleus, metaphase blastomeres were prepared from four-cell stage embryos as described previously (Kwon & Kono 1996). Mouse–pig iSCNT was performed by injecting a cumulus cell nucleus into a zona intact enucleated MII pig oocyte by piezo actuated microinjection as described elsewhere (Wakayama *et al.* 1998). Where necessary, donor cells were fused to enucleated cytoplasts by electrical pulse using an eppendorf multiporator and fusion chamber with a 200 µM electrode gap (Eppendorf, Hamburg, Germany). Sheep and cow oocyte–donor cell couplets were fused by applying two DC pulses of 1.25 KV/cm for 30 µs in 0.3 M mannitol without calcium ions. When pig oocytes were used as cytoplasts, fusion and activation was achieved by two DC pulses of 1.25 KV/cm for 60 µs in 0.3 M mannitol containing calcium. Parthenogenetic pig embryos were produced similarly by activating MII pig oocytes.

Intra-species spindle transfer (SPNT) and inter-species spindle nuclear transfer (iSPNT) in cattle and sheep were carried out using a zona-free method as described above. In place of somatic cell or embryonic blastomeres as nuclear donors, a karyoplast containing the MII spindle removed from cow and sheep oocytes was fused with enucleated cow or sheep oocytes or vice versa, respectively, for intra- and inter-species spindle transfer, using electrical fusion as described above.

Reconstructed intra-species, inter-species, or spindle NT embryos and control sheep and cow oocytes were parthenogenetically activated as described previously (Choi & Campbell 2010, Kelly *et al.* 2010). In brief, oocytes or reconstructed embryos were incubated in H-SOF medium supplemented with 5 µM calcium ionophore (A23187) for 5 min, followed by 5 h incubation in culture medium (C-SOF) supplemented with 10 µg/ml cycloheximide and 7.5 µg/ml of CB at 39 °C in a humidified atmosphere of 5% CO₂ in air. Pig SCNT or iSCNT or MII oocytes were activated by culture in PZM with 10 µg/ml

cycloheximide and 7.5 µg/ml of CB at 39 °C for 5 h in a humidified atmosphere of 5% CO₂ in air. Mouse–pig embryonic iSCNT oocytes, however, were activated in PZM with 10 µg/ml cycloheximide but without CB to allow extrusion of polar body to establish diploid status.

All embryos were then cultured using media and incubation conditions optimal for the cytoplasm origin. Embryos reconstructed using bovine or ovine oocyte as cytoplasm were cultured in modified SOFaaci supplemented with 16 mg/ml fatty acid-free BSA (C-SOF) and embryos from porcine cytoplasm were cultured in PZM-3 with 0.4% BSA at 39 °C in a humidified atmosphere of 5% O₂, 5% CO₂, and 90% N₂ for 6–7 days.

Generation of mouse embryos supplemented with porcine cytoplasm

Female B6D2F1 mice aged 8–10 weeks were superovulated by the administration of 5 IU of pregnant mare serum gonadotropin and hCG at an interval of 48–52 h. Females were placed with B6D2F1 males immediately after hCG injection, and the following morning females were examined for the presence of a vaginal plug as evidence of mating. Mice were killed by cervical dislocation and zygotes were collected from the ampullae of oviducts at 18 h post-hCG. Cumulus cells, if present, were removed by gentle pipetting after a brief exposure to hyaluronidase (300 IU/ml). To produce cytoplasmic hybrid embryos, mouse zygotes were first prepared by removing first and second polar bodies, if present. Then, using a 20 µm blunt micropipette, cytoplasm was aspirated from enucleated activated zona-free pig oocyte and then transferred into the mouse zygotes from which polar bodies were removed. To evaluate the extent to which a mouse zygote can tolerate foreign ooplasm, pig–mouse cytoplasmic hybrids were prepared containing pig cytoplasm 1/10 (SVCH) or 1/5 (LVCH) of the total volume of the mouse zygotes. Volume of pig cytoplasm fused to mouse zygotes was calculated using mathematical formulas for conversion of

surface to volume ratio as described (Liang *et al.* 2009). The pig cytoplasm was electrofused to the mouse oocytes using an AC pulse (2.0 KV/cm for 5 s) to align the couplets followed by two DC pulses of 1.25 KV/cm for 30 µs in 0.3 M mannitol using a multiporator (Eppendorf). The reconstructed cytoplasmic hybrid and control mouse embryos were washed several times in mouse culture medium, KSOM, and then cultured up to 120 h post-hCG in the same medium under mineral oil at 37 °C in a humidified atmosphere of 5% O₂, 5% CO₂, and 90% N₂.

Embryo transfer

Morula–blastocyst stage embryos developed from SVCH or control manipulated embryos at 96 h post-hCG were transferred into the uterus of day 2.5 pseudopregnant females mated to vasectomized males of proven sterility. We transferred a total of 13–20 embryos divided equally between the two oviducts per recipient.

DNA extraction from embryos

DNA samples were isolated from LVCH embryos and control mouse embryos at the zygote (24 h post-hCG), two-cell (48 h post-hCG), and morula–blastocyst stages (96 h post-hCG). The embryos (10/tube) were transferred into a 0.2 ml tube containing 20 µl double distilled water, 100 µg/ml proteinase K, and 0.2% Triton-X 100. The mixtures were incubated at 55 °C for 30 min, 99 °C for 5 min, and then centrifuged at 1200 g for 5 min. The supernatants were diluted 1:5 in PCR-graded water (Roche Diagnostics) and used as templates for PCR or qRT-PCR.

Generation of DNA standards for RT-PCR

The external standards for mouse and porcine mtDNA copy number were generated as described previously (Facucho-Oliveira *et al.* 2007) using gene-specific primers. Briefly, 2 µl

Table 4 Primers used for real-time PCR.

Gene	Forward primers (5'–3')	Reverse primers (5'–3')	Annealing temp. (°C)	Extension time (s)
Porcine <i>mtDNA</i>	CTCAACCCTAGCAGAAACCA	TTAGTTGGTCGTATCGGAATCG	56	20
Mouse <i>mtDNA</i>	CAGTCTAATGCTTACTCAGC	GGGCAGTTACGATAACATTG	53	15
<i>Tfam</i>	GCATACAAAGAAGCTGTGAG	GTTATATGCTGAACGAGGTC	53	15
<i>Polg</i>	GGACCTCCCTTAGAGAGGGA	AGCATGCCAGCCAGAGTCACT	60	15
<i>Polg2</i>	ACAGTGCCTTCAGGTTAGTC	ACTCCAATCTGAGCAAGACC	55	20
<i>mt-Cox1</i>	TTTTCAGGCTTCACCCCTAGATGA	CCTACGAATATGATGGCGAAGTG	63	13
<i>mt-Cox2</i>	TGAAGACGTCCTCCACTCATGA	GCCTGGGATGGCATCAGTT	63	13
<i>Cdx2</i>	AGACAAATACCGGGTGGTGTA	CCAGCTCACTTTTCTCTCTGA	61	10
<i>Gata3</i>	GGGTTCCGATGTAAGTCGAG	CCACAGTGGGGTAGAGGTTG	61	10
<i>Tcfap2c</i>	TGGGTGACGTGTGGATTGG	ACACAACAATTATTTAAGGAGCACGGA	63	13
<i>Tead4</i>	ACGGAGGAAGGCAAGATGTA	CTGGAGACCTGCTTCCTTGT	61	10
<i>Pou5f1</i>	CTCACCCTGGGCTTCTCT	AGGCCTCGAAGCGACAGA	63	13
<i>Nanog</i>	CCTCAGCCTCCAGCAGATGC	CCGCTTGCACTTCATCCTTTG	65	15
<i>Bcl2</i>	CCGGGAGAACAGGGTATGATAA	CCCACTCGTAGCCCCCTCTG	61	17
<i>Hspb1</i>	GTTTCAGATCATGTCAGACC	AACATTCTGGATTCACTACC	56	17
<i>Mfn2</i>	GTGGGCTGGAGACTCATCG	CTCACTGGCGTATTCCACAA	60	15
<i>Slc2a1</i>	CCAGCTGGGAATCGTCGTT	CAAGTCTGCATTGCCCATGAT	61	15
<i>Slc2a3</i>	CTCTCAGGTCACCCAACACTACGT	CCGCGTCCTTGAAGATTCC	61	15
18S RNA	AGAAACGGCTACCATCCAA	CCTGTATTGTTATTTTCGTCACCTACCT	61	15

diluted DNA was combined with 2.5 µl of BD Advantage 2 PCR buffer, 0.5 µl of 50×dNTP mix (10 mM each), 0.5 µl of 50×BD advantage 2 polymerase mix, and 1 µl of primer mix (10 mM). PCR reactions were performed for 40 cycles at 94 °C for 15 s and 68 °C for 45 s (Bio-Rad iCycler; Bio-Rad) and the products were purified from 2% agarose gels using the QiaQuick Gel Extraction kit (Qiagen) according to the manufacturer's instructions and then inserted into pCR8/GW/TOPO TA cloning vector (Invitrogen), transformed in DH5α cells, and plasmid DNA was isolated using GenEute plasmid miniprep kit (Sigma). The plasmid vectors containing mouse and porcine mtDNA were serially diluted 10fold, covering 10⁸ to 10¹ copies/2 µl, for use as standards for RT-PCR.

Reverse transcription for qRT-PCR

Total RNA from embryos was directly converted to cDNA using the FastLane cell cDNA kit (Qiagen) according to the manufacturer's instructions. Briefly, ten morula/blastocyst stage embryos per batch were lysed with 8 µl FCP buffer and genomic DNA was eliminated by gDNA wipeout. The whole 14 µl lysate was then directly reverse transcribed at 42 °C for 45 min. The resulting cDNA was then diluted 1:5 in PCR grade water (Roche Diagnostics) before analysis.

RT-PCR

RT-PCR was performed on a LightCycler 480 (Roche Diagnostics) using 384-well plates. Each 15 µl volume reaction contained 2 µl diluted DNA or cDNA, 7.5 µl of 2X LightCycler 480 SYBR Green I Master Mix (Roche Diagnostics), 330 nM primers, and PCR grade water (Roche Diagnostics; see Table 4). The thermal programme included a 10 min incubation at 95 °C to activate FastStart DNA polymerase, followed by 45 cycles of 95 °C for 10 s, the appropriate annealing temperature (see Table 4) for 15 s and 72 °C for the appropriate extension time with single fluorescence acquisition. Melting curves were performed to ensure gene-specific amplification. All samples were run twice in triplicate and cycle threshold values of the target genes were normalized to external standards for mtDNA copy number or an internal standard 18S rRNA for gene expression analysis. Quantification of mtDNA copy numbers or different gene expression was calculated by Microsoft Excel using the standards as described above or according to the 2^{-ΔΔC_T} method (Schmittgen & Livak 2008). The mtDNA copy numbers were converted to values for a single embryo.

DNA methylation analysis by bisulfite sequencing

The EZ DNA methylation-direct kit (Zymo Research, Orange, CA, USA) was used for bisulfite conversion of DNA. Briefly, ten LVCH and control mouse morula/blastocyst embryos at 96 h post-hCG were digested with proteinase K (provided in the kit) in 20 µl digestion solution for 20 min at 50 °C. The DNA was denatured at 98 °C for 8 min and incubated for 3.5 h at 64 °C with CT conversion reagent, followed by treatment with M-desulfonation buffer for 20 min at room temperature and

elution. Specific regions of *Pou5f1* and *Line1* were amplified as described previously (Bian *et al.* 2009). TA cloning, transformation, and plasmid DNA isolation were performed as described above. The DNA inserted into each plasmid was sequenced using an ABI 373 DNA Sequencer (PE Applied Biosystem, Warrington, UK).

Statistical analysis

Statistical analysis was performed using the Sigma-Stat Software (Jandel Scientific Software, Chicago, IL, USA). Differences in embryo developmental rates and DNA methylation were determined using the χ^2 test. One-way ANOVA was used to determine differences in mtDNA copy number and gene expression between the cytoplasmic hybrid and control mouse embryos. *P* values <0.05 were considered statistically significant differences unless stated.

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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