Characterization of mitochondrial replication and transcription control during rat early development *in vivo* and *in vitro*

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Abstract

In vitro culture (IVC), used in assisted reproductive technologies, is a major environmental stress on the embryo. To evaluate the effect of IVC on mitochondrial transcription and the control of mtDNA replication, we measured the mtDNA copy number and relative amount of mRNA for mitochondrial-related genes in individual rat oocytes, zygotes and embryos using real-time PCR. The average mtDNA copy number was 147 600 (± 3000) in metaphase II oocytes. The mtDNA copy number was stable throughout *in vivo* early development and IVC induced an increase in mtDNA copy number from the 8-cell stage onwards. *Gapd* mRNA levels vary during early development and IVC did not change the patterns of these housekeeping gene transcripts. *Polrmt* mRNA levels did not vary during early development up to the morula stage but increased at the blastocyst stage. IVC induced the up-regulation of *Polrmt* mRNA, one of the key genes regulating mtDNA transcription and replication, at the blastocyst stage. An increase in *mt-Nd4* mRNA preceded the blastocyst-related event observed in nuclear-encoded *Gapd* and *Polrmt*, suggesting that the expression of mitochondrial encoded genes is controlled differently from nuclear encoded genes. We conclude that the IVC system can perturb mitochondrial transcription and the control of mtDNA replication in rat embryos. This perturbation of mtDNA regulation may be responsible for the abnormal physiology, metabolism and viability of *in vitro*-derived embryos.

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Introduction

Rats are the most widely studied experimental animal models in toxicology. In this field, embryo culture methods have become important tools for elucidating the prenatal toxicity of chemicals (Stahlmann *et al.* 1993) and whole embryo culture systems have been developed to assess malformation and embryo toxicity after implantation (Webster *et al.* 1997). However, no *in vitro* screening test has been established during rat early development, partially due to inadequate culture systems for preimplantation stage embryos. Nonetheless, some *in vitro* culture systems have recently been developed for the culture of zygotes through the blastocyst stage in several strains of rats (Miyoshi *et al.* 1997, Zhou *et al.* 2003). These systems could provide not only a beneficial tool for the assessment of environmental stress but also a powerful tool for the development of reproductive technologies in rats.

Optimized *in vitro* culture (IVC) systems for the preimplantation stages of development are critical components for most human and animal assisted reproductive technologies. For instance, it is known

that IVC conditions can have a profound effect on postimplantation development, including physiology, metabolism and viability (Khosla *et al.* 2001, Gardner & Lane 2005). Moreover, reports have shown that the exposure of mouse embryos to suboptimum culture conditions can lead to long-term behavioral effects in the resultant offspring (Ecker *et al.* 2004, Fernandez-Gonzalez *et al.* 2004). On the other hand, a report has shown that embryo culture does not affect the longevity of offspring in mice (Sommovilla *et al.* 2005). Recently, IVC has been shown to be correlated with significant up- or down-regulation, *de novo* induction or silencing of genes critical for fetal and neonatal development (de A Camargo *et al.* 2005, Gardner & Lane 2005, Wrenzycki *et al.* 2005), indicating that the comparison of gene expression patterns between *in vivo* and *in vitro* embryos is a reasonable index to develop IVC systems in different mammals.

Mitochondria play a key role in the physiology of eukaryotic cells during all stage of life including the preimplantation period and their main function is to provide cells with ATP through oxidative phosphorylation

(Bavister & Squirrell 2000, Brenner *et al.* 2004, Cummins 2004, Smith *et al.* 2005). Mitochondria contain their own genome and mitochondrial DNA (mtDNA) encodes 13 essential components of the oxidative phosphorylation pathway, including six NADH dehydrogenase subunits (mt-Nd1 to 6) of Complex I. The remaining genes required for mitochondrial function, more than 200 (Cummins 2004), are transferred to the nuclear genome during the evolutionary process. Consequently, mitochondria rely on nuclear-encoded products not only to produce the remaining oxidative phosphorylation component, but also to provide direct support for general housekeeping tasks and for other important physiological roles (i.e. in apoptosis and steroidogenesis; Smith *et al.* 2005). Although the mechanisms, which regulate the amounts of mitochondrial mRNA and DNA molecules remain elusive, studies in somatic cells have indicated that mtDNA transcription, and the subsequent use of nascent RNA to prime its replication, involves a nuclear-encoded RNA polymerase (Polrmt) and the mitochondrial-specific transcription factors Tfam and Tfb2 (Clayton 2000, 2003, Moraes 2001, Gaspari *et al.* 2004).

Little is known about mtDNA replication and transcriptional events in mammalian oocytes, zygotes and early embryos. The mitochondrial copy number has been reported in oocytes from mice (Piko & Taylor 1987, Thundathil *et al.* 2005), bovines (Smith *et al.* 2005), pigs (El Shourbagy *et al.* 2006) and humans (Steuerwald *et al.* 2000, Reynier *et al.* 2001, May-Panloup *et al.* 2005a) but, to date, there is no report of the mtDNA copy number in rat oocytes. Pioneering studies by Piko & Taylor (1987) showed that the quantity of mtDNA remains stable throughout early development in mice, suggesting an absence of mtDNA replication during preimplantation; however, it has been suggested that there is a very short period of mtDNA synthesis immediately after fertilization in mice, which can be affected by environmental stress (McConnell & Petrie 2004). In an attempt to clarify the control of mitochondrial function, we have recently shown dynamic changes in the transcription patterns of mitochondrial and nuclear genes that encode mitochondrial transcription and replication factors, suggesting that most molecular components are available for mtDNA replication pre-implantation (Thundathil *et al.* 2005). Previous studies clearly indicated the tight coordination of nuclear and mitochondrial OXPHOS transcripts during early development (Taylor & Piko 1995). Moreover, a recent study implied that mitochondria directly influence fertilization outcome and subsequent embryonic development in pigs (El Shourbagy *et al.* 2006).

We hypothesize that IVC, a major environmental stress on embryos used in assisting reproductive technologies, may perturb mitochondrial transcription and the control of mtDNA replication. Since, few reports have focused on improving culture systems for rats, the object of this study is to evaluate the effect of *in vitro* culture on the molecular control of nuclear-mitochondrial interactions during rat

early development. In pursuit of this objective, we measured the mtDNA copy number and relative amount of mRNA for mitochondrial-related genes in individual rat oocytes, zygotes and embryos using real-time PCR.

Materials and Methods

Chemical source

Unless otherwise noted, all chemicals and reagents were purchased from Sigma Chemicals Co.

Animal source

The rats were obtained from Charles River Canada (St-Constant, Québec, Canada) and kept on a 14 h light:10 h dark schedule (dark period peaked at midnight). Female Sprague-Dawley (SD) rats (6–10 week old) were used to collect oocytes and to mate with male Brown Norway rats to obtain fertilized embryos. All animal treatment protocols were approved by the Comité de Déontologie, Faculté de Médecine Vétérinaire, Université de Montréal in accordance with the regulations of the Canadian Council of Animal Care.

Oocyte, zygote and embryo sampling

Oocytes were collected from superovulated females injected with eCG (15 IU, Folligon, Intervet, Ontario, Canada) followed 48 h later by hCG (25 IU, Chorulon, Intervet). Oocytes were collected from oviducts at 14–15 h after hCG injection using Hepes-buffered K modified simplex optimized medium (KSOM) (H-KSOM; Lawitts & Biggers 1993). Cumulus cells were removed by pipetting H-KSOM containing hyaluronidase (20 IU/ml).

Zygotes (1-cell stage) were collected from natural ovulated and superovulated females. Proestrus females selected by vaginal smear (natural ovulation) and an eCG-hCG primed female (superovulation) were caged overnight with fertile males and killed at 1500 h the following day (24 h after hCG injection). Zygotes recovered from superovulated females were cultured using a two-phase chemically defined system (Zhou *et al.* 2003) comprised of culture for the first 18 h in KSOM (Lawitts & Biggers 1993), then in mR1ECM (Miyoshi *et al.* 1997) up to 120 h. *In vitro*-derived early 2-cell embryos, late 2-cell embryos, 8-cell embryos, morulae and blastocysts were selected and sampled at 24, 48, 72, 96 and 120 h of culture respectively. On the other hand, *in vivo*-derived early 2-cell embryos, late 2-cell embryos, 8-cell embryos, morulae and blastocysts were sampled from naturally mated females at 1500 h on day 2, 0900 h on day 3, 0700 h on day 4, 0300 h on day 5 and 1500 h on day 5 respectively (day 1 = the day of sperm detection in vaginal smear). Only morphologically excellent oocytes, zygotes and embryos were washed with PBS containing 0.1% (W/V) polyvinyl

alcohol (PVA), loaded individually into a siliconized 1.5 ml tube with less than 5 µl of the PBS and stored at –70 °C until use.

Isolation of genomic DNA and mRNA

Genomic DNA and mRNA were isolated from each individual oocyte, zygote or embryo with oligo-dt conjugated beads using Dynabeads mRNA Direct Kit (Dyna, Oslo, Norway) according to the manufacturer's protocol with modifications. Briefly, 5 pg rabbit globin mRNA (Sigma) was added to each sample as an external standard. Each oocyte, zygote or embryo was lysed in 100 µl of lysis/binding buffer by gentle vortexing for 10 min and 10 µl of prewashed beads were added to the fluid. After a second gentle vortexing for 10 min, the beads were separated with a magnetic separator (Dyna) and the supernatant was used for the extraction of genomic DNA. mRNA was eluted from the beads by incubation in 10 µl sterile water at 70 °C for 3 min and reverse transcribed (RT) into cDNA using a Sensiscript RT Kit (Qiagen) according to the manufacturer's protocol. Reverse transcriptase was omitted during the RT reaction in the negative controls. The cDNA was purified with 0.8 µg of MS2 carrier RNA (Roche Diagnostics) using MinElute Reaction Cleanup Kit (Qiagen) according to the manufacturer's protocol and eluted twice with 11 µl sterile water. Carrier RNA (333 ng, Qiagen) was added to each sample before genomic DNA extraction (QIAmp DNA mini kit, Qiagen), eluted twice with 50 µl of sterile water and diluted ten times for quantitative PCR. Both cDNA and genomic DNA were used for quantitative PCR on the day of extraction to avoid degradation.

Primer design

Primer pairs for the relative quantification of gene expression and absolute quantification of mtDNA copy number are listed in Table 1. Primers of *Gapd*, *Polrmt* and *mt-Nd4* were designed using published rat cDNA sequences (GenBank, NM_017008, XM_216836 and

NC_001665 respectively). Primers for the quantification of mtDNA were designed from homologous regions of 16S rRNA genes in published mitochondrial sequences of rats (NC_001665).

Absolute quantification of mitochondrial DNA copy number

Quantification standards were prepared according to our previous report (Thundathil *et al.* 2005). A long 1333 bp fragment between *16SrRNA* and *ND1* region of mtDNA was amplified from rat liver by PCR using primer pair 5'-ATA ATC ACT TGT TCC TTA ATT AG-3' and 5'-CTA ATT CTG ATT CTC CTT CTG-3'. The PCR product was extracted from the gel using QIAquick Gel Extraction Kit (Qiagen) and cloned using QIAGEN PCR Cloning Kit. The plasmid DNA was purified using QIAprep Spin Miniprep Kit (Qiagen), quantified by spectrophotometer and diluted at 1×10^7 copies/µl. To simulate embryo samples, 10 µl of standard stock was extracted and serially diluted for use in the standard curve. Real-time fluorescent monitored quantitative PCR was performed in a LightCycler (Roche) as described in our previous report (Thundathil *et al.* 2005) with modifications. The PCR program employed an initial step of 95 °C for 5 min followed by 45 cycles of 3 s at 94 °C for denaturation, 2 s at 62 °C for annealing, and 10 s at 72 °C for elongation. The melting curve of the amplified product was achieved at 95 °C for 5 s, 55 °C for 5 s, and 99 °C for 0 s. Cooling was done at 40 °C for 10 s. Temperature transition rates were set at 20 °C/s except for the final melting step (0.1 °C/s). A fluorescent signal was acquired continuously at the end of elongation for quantification and the final melting step to identify the PCR product. Premix for quantitative PCR was prepared from LightCycler FastStart DNA Master SYBR Green I (Roche). The premix consisted of 5.4 µl of PCR grade water, 1.6 µl of 25mM MgCl₂, 2 µl of hot start reaction mix, and 1 µl of 10 mM primer pair and 10 µl of DNA template was added to 10 µl of premix for PCR reaction.

Table 1 Primers for real-time PCR.

Purpose	Gene	Sequence	Product size (bp)
Relative quantification of gene expression	<i>Gapd</i>	CAT TGT TGC CAT CAA CGA CC GTA GAC TCC ACG ACA TAC TC	210
	<i>Polrmt</i>	CCT ACA CTG CTA CAG GAA G GCA AGG ATC GTA GTG TCT C	242
	<i>mt-Nd4</i>	ATA ATT ATA ACT AGC TCA ATC TGC TCG TAG TTG GTG TTT GCT AG	200
External control of gene expression	<i>Rabbit α -1-globin^a</i>	GCA GCC ACG GTG GCG AGT AT GTG GGA CAG GAG CTT GAA AT	257
Absolute quantification of mtDNA copy number	<i>mtDNA</i>	TTG ATC AAC GGA CCA AGT TAC CTG GAT TGC TCC GGT CTG A	185

^aWrenzycki *et al.* (1999).

Relative quantification of mRNA amounts

The standard was prepared with mRNA from rabbit *globin* (Sigma), reverse transcribed, purified, amplified by PCR, extracted from the gel and cloned. Plasmid DNA was purified, quantified and serially diluted to 1000, 100, 10, 1, 0.1, 0.01 and 0.005 fg/μl. With the exception of denaturation at 95 °C for 5 s and annealing at 58 °C for 5 s, quantitative PCR was performed as described above. cDNA templates for individual embryos were recovered and used for amplification of the *globin* standard, *Gapd*, *Polrmt* and *mt-Nd4*. A premix was prepared consisting of 8.9 μl of PCR grade water, 1.6 μl of 25 mM MgCl₂, 0.5 μl of DMSO, 2 μl of hot start reaction mix and 1 μl of 10 mM primer pair.

Cell number count of blastocyst

Some blastocysts were fixed with 10% (v/v) neutral-buffered formalin for 15 min and permeabilized overnight with PBS containing 0.5% (w/v) Triton X-100 and 0.1% (w/v) polyvinyl alcohol. Blastocysts were stained with 0.1 μg/ml of Hoechst dye 33342 (Sigma) and nuclei were counted under a fluorescent microscope.

Statistical analysis

All data were analyzed by one-way ANOVA followed by Tukey honest significance differences and a probability of *P* < 0.05 was considered statistically significant.

Results

Development of in vitro cultured zygotes

To assess the efficiency of our culture system, we recorded the developmental rate of 1-cell stage zygotes at different periods of *in vitro* culture (Table 2). Almost all zygotes had already cleaved by 24 h and remained at the 2-cell stage until 48 h of culture. At 72 h of culture, more than 90% of cultured zygotes had developed to either the 4-cell or non-compacted 8-cell stage. Most of the 4-cell and 8-cell stage embryos developed to the early or non-expanded blastocyst stage at 96 h of culture. Maximum blastocyst development (81%) was obtained at 120 h of culture, indicating that the culture system was

able to support *in vitro* development from zygote to blastocyst efficiently.

Comparisons between *in vitro*- and *in vivo*-derived embryos should preferably be made among counterparts at similar stages of development. Since, preliminary experiments had indicated that *in vivo* embryos were further advanced in development at fixed time points, adjustments to the timing of collection were required to obtain similar embryo stages for comparison. Therefore, we examined the developmental rate of *in vivo* embryos by continuous sampling throughout preimplantation (data not shown) and brought forward the sampling time to obtain the morphological stages (late 2-cell, 8-cell, morula and blastocyst stage embryos) obtained *in vitro*. These results clearly show that embryos derived from our *in vitro* culture system cleave at a slower rate than *in vivo* embryos. The cell number of cleavage stage embryos can be readily assessed by live microscopy, whereas morula (data not shown) and blastocysts derived *in vivo* and from *in vitro* cultures were assessed by live microscopy (Fig. 1A and B) and after fixation and DNA staining to count the nuclei number (Fig. 1C and D). Blastocysts derived *in vitro* were morphologically different from those derived *in vivo*. Blastocysts derived *in vitro* were expanding and in some cases already hatching at the time of harvesting (Fig. 1B), whereas blastocysts derived *in vivo* had an oval shape and a large perivitelline space (Fig. 1A). Nonetheless, the total cell number of blastocysts derived *in vitro* (37.8 ± 1.2 , *n* = 52, Fig. 1D) was remarkably similar to that derived *in vivo* (38.0 ± 1.5 , *n* = 26, Fig. 1C), indicating that the molecular parameters (mtDNA copy number and mRNA abundance) could be compared among the *in vivo* and *in vitro* groups.

Mitochondrial DNA copy number of in vivo and in vitro embryos

To verify whether the culture system used to obtain embryos *in vitro* had an effect in the mechanisms controlling mtDNA levels during early embryogenesis, we quantified the mtDNA copy numbers of *in vivo*- and *in vitro*-derived embryos from zygote to blastocyst stages (Fig. 2). The mean mtDNA copy number in rat metaphase II oocytes was 147 600 (± 3000 , *n* = 11) with small oocyte variation (123 400–156 900). Since,

Table 2 Developmental stage of cultured rat zygotes at sampling.

Duration of culture (h)	No. of replicates	No. of ova observed	Developmental stage						
			1-cell (%)	2-cell (%)	4-cell (%)	8-cell (%)	Morula (%)	Blastocyst (%)	Fragmented (%)
24	4	41	2	98					
48	4	44	2	95	2				
72	5	108	1	4	52	42			2
96	5	94	1	1	6	9	51	28	4
120	6	161	2	4	2	1	8	81	3

Figure 1 Optical (A and B, $\times 20$) and fluorescence (C and D, $\times 40$) microscopic images of *in vivo*-derived (A and C) and *in vitro*-derived (B and D) rat blastocysts.

in vitro cultured embryos were recovered from eCG-stimulated females, we compared the mtDNA copy number per zygote between naturally ovulated (NO) and superovulated (SO) females. There was no significant difference in the mean mtDNA copy number ($\pm$ S.E.M.) between the two groups (NO: $127\,134 \pm 4780$, $n=14$, SO: $124\,869 \pm 4962$, $n=14$). From these results, we conclude that SO does not affect the mtDNA copy number in zygotes used for *in vitro* culture. Our next step was to compare the mtDNA copy number between *in vivo*- and *in vitro*-derived embryos from the early 2-cell to blastocyst stages. There were no significant differences at early 2-cell (*in vivo*: $137\,018 \pm 5692$, $n=11$; *in vitro*: $139\,478 \pm 4500$, $n=9$), late 2-cell (*in vivo*: $149\,833 \pm 7870$, $n=12$; *in vitro*: $150\,860 \pm 7255$, $n=10$) and 8-cell stages (*in vivo*: $141\,882 \pm 7676$, $n=11$; *in vitro*: $164\,930 \pm 7861$, $n=10$); however, significant differences were observed at the morula (*in vivo*: $128\,995 \pm 5224$, $n=13$; *in vitro*: $166\,222 \pm 3733$, $n=9$) and blastocyst stages (*in vivo*: $129\,400 \pm 10\,499$, $n=14$; *in vitro*: $166\,416 \pm 11\,829$, $n=14$), indicating that the *in vitro* culture environment can

affect the control of mtDNA copy number during the later stages of preimplantation development. Finally, we also compared the mtDNA copy number among the different developmental stages within the *in vivo*

Figure 2 Mitochondrial DNA copy number in rat oocytes, zygotes (derived from NO and SO) and embryos (derived *in vivo* and *in vitro*). Data were collected from at least four reactions. Developmental stages not connected by the same letter are significantly different ($P < 0.05$).

(including NO-derived zygotes) and *in vitro* (including SO-derived oocytes and zygotes) groups. In the *in vivo* group, the mtDNA copy number was stable ($P>0.05$) throughout early development, i.e. from the zygote to blastocyst stage. On the other hand, the mtDNA copy number of *in vitro*-derived 8-cell, morula and blastocyst embryos was significantly higher than in zygotes ($P<0.05$). These results confirm that the *in vitro* culture conditions induce an increase in the mtDNA copy number from the 8-cell stage onwards.

Transcript amounts of *in vivo* and *in vitro* embryos

To determine whether the increase in the mtDNA copy number of *in vitro*-derived embryos was related to transcriptional alterations, we quantified the relative mRNA amounts of glyceraldehydes-3-phosphate dehydrogenase (*Gapd*), nuclear-encoded mitochondrial RNA polymerase (*Polrmt*), and NADH dehydrogenase subunit 4 (*mt-Nd4*) in oocytes (SO $n=8$), zygotes (NO $n=5$, SO $n=6$), early 2-cell (*in vivo* $n=6$, *in vitro* $n=5$), late 2-cell (*in vivo* $n=6$, *in vitro* $n=6$), 8-cell (*in vivo* $n=9$, *in vitro* $n=7$), morula (*in vivo* $n=5$, *in vitro* $n=6$) and blastocyst (*in vivo* $n=5$, *in vitro* $n=5$) -stage embryos. The amount of *Gapd* mRNA remained low and stable until the 8-cell stage but increased significantly in morula (*in vitro* embryo) and blastocyst stages (*in vivo* and *in vitro* embryos; Fig. 3). There was no significant difference in the amount of *Gapd* mRNA between *in vivo* and *in vitro* embryos during early development. These results indicate that the expression of *Gapd* varies during early development and the culture system used in these experiments does not change the patterns of expression of this housekeeping gene.

The relative amount of *Polrmt* mRNA was measured to determine whether nuclear-mitochondrial interactions were disturbed by the *in vitro* systems used to culture rat preimplantation-stage embryos (Fig. 4). As for *Gapd*,

Figure 3 Relative amount of *Gapd* mRNA in rat oocytes (derived from SO), zygotes (derived from NO and SO) and embryos (derived *in vivo* and *in vitro*). Data were collected from at least three reactions. Developmental stages not connected by the same letter are significantly different ($P<0.05$).

Figure 4 Relative amount of *Polrmt* mRNA in rat oocytes (derived from SO), zygotes (derived from NO and SO) and embryos (derived *in vivo* and *in vitro*). Data were collected from at least three reactions. Developmental stages not connected by the same letter are significantly different ($P<0.05$).

Polrmt mRNA levels did not vary significantly during early development up to the morula stage but increased significantly in blastocysts. Interestingly, a slight non-significant increase in *Polrmt* was observed at the late 2-cell stage, which had disappeared by the 8-cell stage. Moreover, mRNA amounts in blastocysts derived *in vitro* were twice as high as in *in vivo*-derived blastocysts ($P<0.05$), indicating that the *in vitro* culture system induces an unusual up-regulation of *Polrmt*.

The levels of *mt-Nd4* mRNA were assessed to determine whether the *in vitro* environment affected the regulation of mitochondrial-encoded transcripts during early development (Fig. 5). The amount of *mt-Nd4* mRNA was relatively abundant throughout early development and the expression pattern was different from *Gapd* and *Polrmt*. For *in vivo* embryos, the amount of *mt-Nd4* mRNA was increased at the late 2-cell stage, slightly decreased at the 8-cell stage, and remained stable up to the blastocyst stage. The only significant difference was observed between early and

Figure 5 Relative amount of *mt-Nd4* mRNA in rat oocytes (derived from SO), zygotes (derived from NO and SO) and embryos (derived *in vivo* and *in vitro*). Data were collected from at least three reactions. Developmental stages not connected by the same letter are significantly different ($P<0.05$).

late 2-cell stages. On the other hand, *mt-Nd4* mRNA of *in vitro* embryos was significantly increased at the late 2-cell stage and remained stable until the morula stage with a slight decrease at the blastocyst stage. Interestingly, the amount of *mt-Nd4* mRNA in *in vitro* embryos was significantly higher than that in *in vivo* embryos at the 8-cell and morula stages of development. These results indicate that the increase in mitochondrial transcription precedes the later blastocyst-related event observed in the nuclear-encoded mitochondrial and housekeeping genes analyzed in this study. Moreover, increases in mitochondrial transcripts in the *in vitro* environment were larger and remained elevated during the more advanced stages of pre-implantation development.

Discussion

Artificial reproductive technologies are often utilized in the clinical setting to circumvent infertility problems, requiring the use of suitable experimental models to optimize *in vitro* culture systems that support early embryogenesis. Although mouse embryos have been widely used in the past, it is essential that another easily accessible laboratory species, i.e. the rat, be also examined to widen our understanding of conserved developmental requirements in other mammals. Due to the critical role mitochondria play on embryo survival, we have focused our attention on the effects of *in vitro* culture systems on this organelle and its interaction with the nucleus. Using an efficient culture system that supports the development of rat zygotes to the blastocyst stage, we demonstrate here that the *in vitro* environment significantly affects both mtDNA copy number and mitochondrial transcript abundance during preimplantation.

Previous reports have shown that rat zygotes, harvested at the syngamy-stage (14–16 h postovulation), cultured *in vitro* using a two-phase chemically defined system develop efficiently to the blastocyst stage (Zhou *et al.* 2003). The zygotes used in our study, which recovered at 24 h after hCG, were in the late pronuclear stage at recovery and, because ovulation occurs between 10 and 12 h after hCG (Holland *et al.* 2001), supposed to be comparable to zygotes at 12–14 h postovulation. Although our *in vitro* culture system provided enough embryos for research, the developmental speed was slower than *in vivo* and delayed development was observed from the 4-cell stage onwards. Since, genome activation of rat embryos occurs in the late 2-cell stage (Zernicka-Goetz 1994), *in vitro* embryos may have problems activating the transcription of the embryonic genome leading to the morphology of the resultant blastocysts being different from *in vivo* blastocysts. *In vitro* blastocysts raised in mR1ECM, which includes polyvinylalcohol instead of BSA (Miyoshi *et al.* 1997), may lead to the hardening of the zona pellucidae

similarly to rat oocytes matured *in vitro* in serum-free medium (Zhang *et al.* 1991), which could prevent the hatching embryos cultured *in vitro* and cause morphological differences at the blastocyst stage. Therefore, although this culture system is capable of producing fertile offspring after embryo transfer (Zhou *et al.* 2003), we think that the system needs to be refined further.

To our knowledge, this is the first report of mtDNA copy number in rat oocyte and embryo. The mtDNA copy number in rat matured/ovulated oocyte in our result is close to that in mouse oocyte using dot hybridization (Piko & Taylor 1987) or real-time quantitative PCR (Steuerwald *et al.* 2000, Thundathil *et al.* 2005). Ovarian hyperstimulation does not change the mtDNA copy number in metaphase II oocytes and the resultant embryos. This is a natural outcome, because fully grown germinal vesicle oocytes, responsible for eCG, have already accomplished mtDNA replication (Barritt *et al.* 2002). In bovine oocytes, we observed a slight decrease in the mtDNA copy number during the *in vitro* maturation process (Smith *et al.* 2005).

In the *in vivo* rat embryos, the mtDNA copy number was stable throughout early development, suggesting no replication of mtDNA during this period. However, IVC from the zygote stage induced an increase in mtDNA copy number from the 8-cell stage onward, suggesting that IVC can affect mtDNA replication during early development. A constant mtDNA copy number during *in vivo* early development is observed in mice (Piko & Taylor 1987, Thundathil *et al.* 2005) and Piko & Taylor (1987) suggested an absence of mtDNA replication during this period. On the other hand, we observed a significant increase in mtDNA copy number in *in vitro*-produced expanding and hatching blastocysts in bovines (Smith *et al.* 2005). Increasing mtDNA copy number in *in vitro* embryos can be interpreted as the following two possibilities: (1) accelerated mtDNA replication, which should occur after implantation; in mice, mtDNA replication is known to resume after the egg cylinder stage (Piko & Matsumoto 1976, Ebert *et al.* 1988); (2) failure of mtDNA turnover immediately after fertilization. Recently, it was reported that there is a very short period of mtDNA synthesis from the 1-cell to 2-cell stage, which can be affected by environmental stress in mice (McConnell & Petrie 2004). Further, May-Panloup *et al.* (2005a, 2005b) observed a decrease of mtDNA copy number during 2-cell to morula stage and the following increase at blastocyst stage in *in vitro* produced bovine embryo. Overall, mtDNA copy number is expressed as a result of balance between the synthesis and the degradation of mtDNA and *in vitro* production procedure including IVC could impair the balance.

The expression of *Gapd* as a housekeeping gene increased in the latter part of early development and IVC did not change the patterns of *Gapd* transcripts. This expression pattern agrees with that in *in vivo* mouse

embryos (Thundathil *et al.* 2005), *in vitro* mouse embryos (Jeong *et al.* 2005) and *in vitro*-produced bovine embryos (Robert *et al.* 2002). The expression of *Gapd* could be a useful internal control not to compare between stages but for fixed stages. With the exception of *HistonH2a* in bovine embryos (Robert *et al.* 2002), no gene is known, which fulfils all the criteria throughout early development. Thundathil *et al.* (2005) reported that the expression pattern of *Gapdh*, *Actb* and *HistonH2A* in mouse embryo varied significantly during the period of preimplantation development. *Gapd* is an enzyme, which catalyzes an important energy-yielding step in carbohydrate metabolism and the expression of the housekeeping gene, including *Gapd*, is necessary for cell maintenance and proliferation. A suboptimal environment, which perturbs the expression of the housekeeping gene can lead directly to embryonic death. Embryos at the same stage contain the same amount of *Gapd* mRNA despite their *in vivo* or *in vitro* origin.

Polrmt mRNA levels were relatively low and did not vary during early development up to the morula stage, but IVC increased the levels of *Polrmt* mRNA, one of the key genes regulating mtDNA transcription and replication, at the blastocyst stage, more than twofold. *In vitro* procedures used in the production of fertilized and somatic nuclear transfer bovine embryos have been shown to be correlated with significant up- or down-regulation, *de novo* induction or silencing of genes critical for undisturbed fetal and neonatal development (Wrenzycki *et al.* 2005). It is known that IVC causes down-regulation more frequently rather than up-regulation on the global pattern of gene expression in mouse embryos and most of this up-regulation was less than twofold (Rinaudo & Schultz 2004); however, the expression pattern of *Polrmt* did not correspond completely with the change of mtDNA copy number. Mitochondrial RNA polymerase carries out the central activity of mitochondrial gene expression and, by providing RNA primers for replication, is also implicated in the maintenance and propagation of the mitochondrial genome (Tiranti *et al.* 1997). With *in vivo* embryos, a slight increase of *Polrmt* mRNA at the late 2-cell stage might be related to increased transcription of *mt-Nd4* and further slight increase at the blastocyst stage might trigger mitochondrial replication after the egg cylinder stage (Piko & Matsumoto 1976, Ebert *et al.* 1988). A subtle difference in the amount of *Polrmt* mRNA between *in vivo* and *in vitro* at the late 2-cell stage might cause a difference in the mtDNA copy number at morula and blastocyst stages. We speculate that small amounts of *Polrmt* mRNA could be sufficient for mitochondrial transcription and replication, or a positive feedback system for gene expression (Cross & Tinkelenberg 1991) could explain the expression pattern of *Polrmt*. The first transcription and replication of mtDNA occurred by accumulated *Polrmt* mRNA during oogenesis and consumption of this

mRNA might stimulate *de novo* synthesis at the 2-cell stage; however, the unusual up-regulation of *Polrmt* mRNA in *in vitro*-derived blastocysts might significantly affect mitochondrial replication and transcription after implantation. On the other hand, for the expression pattern of *Polrmt*, the result eliminated the late 2-cell stage as a comparison, *in vivo* rat embryos are similar to *in vivo* mouse embryos (Thundathil *et al.* 2005); however, the increase of *Polrmt* mRNA in mice is more evident than in rats. Transcription in rat mtDNA might be less active than in mouse mtDNA. The *Polrmt* mRNA should increase at the same or the following stage in, which increased expression of promoters for *Polrmt*, like *NRF1* (May-Panloup *et al.* 2005b) and *Tfam* (May-Panloup *et al.* 2005b, Thundathil *et al.* 2005), are observed. First, we tried to analyze expression pattern of *Tfam*, a key regulator for mitochondrial replication and transcription, which is essential for embryonic development (Larsson *et al.* 1998). Although *Tfam* mRNA was detectable in rat oocyte, the relative amounts were too low to get stable result (data not shown). Therefore, we changed the target gene to evaluate mitochondrial replication and transcription from *Tfam* to *Polrmt*.

The amount of *mt-Nd4* mRNA was relatively abundant throughout early development and the mitochondrial-encoded *mt-Nd4* gene could be actively transcribed from the 2-cell stage. In mice, it is reported that mitochondrial transcripts for *Cox* subunits are 30–50-fold more abundant than those derived from nuclear genes during early development (Taylor & Piko 1995). Further, it is reported that the gene expression of nuclear-encoded *Cox* subunits, *Cox5a*, *5b*, and *6b1*, is not required for embryo developmental events up to the blastocyst stage in mice (Cui *et al.* 2006). The abundance of *mt-Nd4* mRNA can be interpreted as a result of this over-expression in mitochondrial-encoded genes. (Hsieh *et al.* 2004) thought that polycistronic mtDNA transcripts are consistent, with different mtRNA expression levels showing the same pattern in oocytes. This could explain why the expression pattern of *mt-Nd4* in *in vivo* rat embryos is similar to that of other mitochondrial-encoded genes (*COX I* and *COX II* (Taylor & Piko 1995), *mt-Nd2* and *mt-Nd6* (Thundathil *et al.* 2005)), in *in vivo* mouse embryos; however, the increase of mitochondrial genes in mice (Taylor & Piko 1995, Thundathil *et al.* 2005) is higher than in rats. An increase in *mt-Nd4* mRNA preceded the blastocyst-related event observed in nuclear-encoded genes and alterations induced by the *in vitro* environment were observed earlier during preimplantation development. This preceding increase could be related to import into mitochondria for the next step. Import is carried out by a complex, ATP-dependent transport system, followed by cleavage of the leader peptide, which eventually produces mature, functional protein (Neupert *et al.* 1990). The expression of mitochondrial-encoded genes might be controlled differently from nuclear-encoded genes. In this experiment, we saw a decreased amount of

mt-Nd4 RNA in *in vitro*-derived blastocysts despite the normal morphology with an expanded blastocoel cavity and a clear inner-cell-mass and normal expression level of *Gapd* as a housekeeping gene. This could be a symptom of cytoplasmic problems connected to embryonic death after implantation in *in vitro*-derived embryos.

PVA, one of the components in mR1ECM, might have affected the gene expression pattern of cultured embryo in the present study. Although modified R1ECM containing PVA instead of BSA is a common medium for the culture of rat embryo, replacement of BSA with PVA in culture media is known to impair hatching of blastocyst in mice (Biggers *et al.* 1997) and to affect amount of developmentally important gene transcripts in bovine blastocyst (Wrenzycki *et al.* 2001). Therefore, PVA could affect gene expression of rat embryos directly or indirectly via morphological changes related with hatching.

Recently, (Alizadeh *et al.* 2005) demonstrate the selective degradation of specific mRNAs after fertilization in mice. The amounts of mRNAs described in this study in zygotes were comparable to those in oocytes. It is possible that none of these mRNAs suffer selective degradation just after fertilization. Studies about the degradation of mRNA, *de novo* nascent mRNA synthesis, and resulting protein synthesis will give further insight into mitochondrial replication and transcription control during early development. Further, studies on mitochondrial distribution and the related proteins are needed to clarify the mitochondrial replication and transcription in mammalian early development.

From these results, the IVC system can perturb mitochondrial transcription and the control of mtDNA replication in mammalian embryos. This perturbation of mtDNA regulation may be responsible for the abnormal physiology, metabolism and viability of *in vitro*-derived embryos. Analysis of the gene expression pattern will be a good tool to develop an IVC system for reproductive technology in animals and humans.

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