Embryonic gene activation in in vitro produced embryos of the domestic cat (Felis catus)

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Abstract

Accurate embryonic gene activation (EGA) is essential for the embryo’s developmental potency and reflects the quality of in vitro produced embryos. To describe the dynamic and temporal patterns of EGA in the cat, the mRNA expression of developmentally important genes (DNA methyltransferases 1 and 3A, DNMT1 and DNMT3A; gap junction protein α 1, GJA1; transcription factor octamer 4, POU5F1 (OCT4); insulin-like growth factor (IGF) 1 and 2 receptors, IGF1R and IGF2R) was examined by RT-PCR techniques in preimplantation embryos obtained after in vitro maturation and IVF. Furthermore, influences of ICSI and sperm cryopreservation on the relative mRNA abundance in 4–5-days-old morulae were analyzed. Total RNA was obtained from immature and matured oocytes, 2-cell embryos, 4-cell embryos, and 8–16-cell embryos, morulae, and blastocysts. RNA was transcribed into single-stranded cDNA by reverse transcriptase. After amplification, a nonfelid standard RNA was used for semiquantitative analysis. Our results showed an increase in transcript abundance from the matured oocyte to the 2-cell embryo for all examined genes except for IGF2R, indicating that, in vitro, the embryonic genome is activated shortly after fertilization. However, the activation pattern varied markedly between the different genes. We also found different patterns of mRNA expression for the examined genes in morulae produced either by IVF or ICSI, and using fresh or cryopreserved sperm. Owing to high variations within the single groups of compared morulae, we were able to observe only a tendency toward higher relative mRNA expression in embryos derived by IVF with fresh sperm in comparison to all other groups.


Introduction

Owing to habitat loss and poaching most wild felid species are threatened with extinction in their native ecosystems, and captive breeding efforts offer the most likely method of ensuring survival. Assisted reproductive technologies (ART) such as artificial insemination, in vitro embryo production (IVP), and cryopreservation of germ cells and embryos are gaining importance as tools to support feline reproduction in captivity and preserve existing genetic diversity. Owing to similarities between the domestic cat (Felis catus) and wild felid species, the former provides a reliable and widely used model organism for development of ARTs in all felids. In the past 20 years, numerous studies have been performed in domestic cats investigating in vitro oocyte maturation, IVF (Johnston et al. 1989, Goodrowe et al. 1991, Jewgenow et al. 1997, Ringleb et al. 2004), influences of ovarian status and season on the oocyte maturation or embryonic developmental potency (Spindler & Wildt 1999, Freistedt et al. 2001), as well as in vitro embryo culture conditions (Herrick et al. 2007) and embryo cryopreservation (Gomez et al. 2003). Although dramatic improvements have been made for in vitro techniques, artificially produced embryos still demonstrate lower developmental competence than in vivo embryos (Roth et al. 1994). Thus, an impact of in vitro culture on the embryo’s developmental competence must be assumed.

In other mammals it was shown that in vitro production of embryos might have an impact on embryo and offspring quality (Hasler et al. 1995). Most obvious were alterations in birth weights after embryo transfer: while there was a decrease in human and mice, cattle and sheep showed increased weights in newborns (De Rycke et al. 2002). To reduce the risk of embryonic failures as a result of in vitro procedures, several techniques for assessing embryonic quality have been developed.

Commonly used criteria for assessing embryonic developmental competence include morphology, habitus, and cleavage timing. Recent improvements in the sensitivity of molecular methods facilitated the application of mRNA abundance and gene expression patterns as powerful tools to evaluate embryonic quality and to compare embryos of different origins. Examinations of the relative abundance of mRNA were conducted primarily in cattle or mice (Lee et al. 2001, Corcoran et al. 2006, Wrenzycki et al. 2006, Nowak-Imlalek et al. 2008). The comparison of specific mRNA values demonstrated dramatic alterations between in vivo and...
in vitro produced embryos (Wrenzycki et al. 1996, Lee et al. 2001, Lonergan et al. 2003, Corcoran et al. 2006). Furthermore, slight alterations of culture medium such as supplementation with fetal bovine serum (FBS), BSA, or polyvinyl pyrollidone (PVP) during maturation and estrous cow serum or polyvinyl alcohol during embryonic development affected transcription levels (Wrenzycki et al. 1999, Warzych et al. 2007).

In bovine, determination of mRNA abundance during early embryonic development is now accepted as an appropriate quality marker for culture conditions and in vitro techniques (Wrenzycki et al. 1996, Warzych et al. 2007, Wrenzycki 2007, Nowak-Imialek et al. 2008). Therefore, we aimed to identify representative genes that could be used for the characterization of embryonic gene activation (EGA) in domestic cat embryos.

For this study, we chose genes known to be essential for early embryogenesis and/or were already used as marker genes for embryo quality in other species (Wrenzycki et al. 1996, 2006, Lonergan et al. 2003, Warzych et al. 2007, Magnani & Cabot 2008). Thus, the products of the selected genes are involved in epigenetic modifications (DNA methyltransferases 1 and 3A, DNMT1 and DNMT3A), intercellular communication (gap junction protein α1, GJA1), transcription regulation (octamer-binding transcription factor 4, POU5F1 (OCT4)), and growth factor reception (insulin-like growth factors 1 and 2 receptors, IGF1R and IGF2R) as well as cell structure formation (housekeeping gene β-actin, ACTB).

To assess the temporal pattern of gene expression in oocytes and early stages of embryonic development, feline preimplantation embryos were produced by in vitro maturation of ovarian oocytes followed by fertilization with fresh epididymal sperms. All stages of in vitro development were subjected to a semiquantitative RT-PCR assay for the selected genes. In addition, we analyzed the impact of fertilization method (in vitro versus ICSI) and sperm source (fresh versus frozen–thawed) on early embryonic gene expression by comparing the relative mRNA abundance of the selected genes in 4–5-days-old morulae.

### Results

#### Sequence analysis

Owing to the lack of sequence information regarding most of our genes of interest, amplification occurred with primers designed based on homologous sequences of nonfelid species (Table 1). Figure 1 shows alignments of obtained sequences with the corresponding cat whole genome shotgun (WGS) sequence (accession AACG00000000). We found no discrepancies for DNMT1 (GQ496518), GJA1 (GQ500999), and IGF2R (GQ484231), whereas the sequences for POU5F1 (GQ484232) and IGF1R (GQ484230) contained one transition and one transversion respectively and for DNMT3A (GQ500998) two deletions and one transition.

**Table 1** Sequence references, primer sequences, and length of each amplified fragment.

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Examination of the temporal pattern of relative mRNA abundance

The temporal pattern of relative mRNA amounts for the seven examined gene sequences is represented in Fig. 2. Maturation in vitro caused a loss of mRNA in all examined genes with the greatest decrease in IGF2R (44%). After fertilization, all genes, except IGF2R, were characterized by a steady increase in mRNA transcripts from matured oocyte through the first two embryo divisions. Beyond the 4-cell stage, however, expression patterns were found to be very gene specific (Fig. 2): DNMTs as well as ACTB mRNA levels increased during development to 8–16-cell embryos, whereas POU5F1, GJA1, and IGF1R mRNA amounts stagnated during this period. With further progress to the morula stage, POU5F1, GJA1, and ACTB mRNA showed increasing levels, whereas DNMT1, DNMT3A, and IGF1R mRNA proportions remained constant. IGF2R mRNA abundance exhibited no change during development from mature oocyte to morula. During blastocyst formation, relative mRNA levels strongly increased for all genes including IGF2R with the exception of IGF1R and GJA1. The IGF1R mRNA level remained constant whereas the GJA1 mRNA level decreased during development from morulae to blastocysts.

Comparison of the relative mRNA amounts in embryos of different origins

The relative mRNA amounts in morulae of different origins (IVF versus ICSI and fresh spermatozoa versus cryopreserved spermatozoa) are presented in Fig. 3. Generally, mRNA expression patterns differed between all examined groups and genes. However,
because of the high variation within single groups, differences were mostly not significant by two-factor ANOVA. The only significant effect found was related to the fertilization method: IVF-produced morulae showed higher relative DNMT1 mRNA amounts than ICSI morulae (F(1, 16) = 5.406, P = 0.034). In addition, morulae derived by IVF with fresh sperm expressed the highest levels of DNMT1 mRNA whereas ICSI embryos produced with cryopreserved sperm cells expressed the lowest relative DNMT1 mRNA amount (Fig. 3A). Parthenogenetic embryos showed relative DNMT1 mRNA amounts twice as high as fresh sperm/IVF embryos (data not shown).

DNMT3A mRNA levels were similar in three groups (fresh and cryopreserved sperm/IVF and fresh/ICSI), only ICSI using cryopreserved sperm caused a reduced expression of DNMT3A (Fig. 3B). Again, the parthenogenetic embryos had the highest levels (data not shown). Regarding GJA1, POU5F1, IGF1R, and ACTB mRNA levels, embryos produced by IVF with fresh sperm were characterized by the highest levels of expression whereas the other three groups (IVF with cryopreserved sperm and both ICSI groups) showed markedly lower levels of mRNA (Fig. 3C–E and G). For IGF2R (Fig. 3F), the variation of expression levels was too high for estimation of trends, likely because of relatively low mRNA amounts.

**Discussion**

This is the first study that describes the pattern of EGA in a nonlaboratory and noncommercial species. The evaluation of preimplantation embryonic quality not only by morphological criteria but also by expression characteristics of marker genes that play crucial roles during early embryonic development is very important to monitor and improve IVP in felid species.

The temporal pattern of EGA is known to be species specific. Autoradiography experiments have shown the initial uptake of [3H]uridine at the 4-cell stage in pig (Kopecky 1989), 5–8-cell stage in domestic cat (Hoffert et al. 1997), and at the end of the 8-cell stage in cattle embryos (Camous et al. 1986, Kopecky et al. 1989). This onset of transcription coincides with the specific developmental block after treatment with α-amanitin, a transcriptional inhibitor (Telford et al. 1990). It is
assumed that the developmental block induced by α-amanitin occurs at the time of major EGA, also known as maternal to embryonal transition (MET), when embryonic transcripts become inevitable for further development (Memili et al. 1998, Memili & First 2000). Embryos are able to cleave until a specific stage consuming mainly maternal mRNA and proteins. At the time of MET, however, they fail in further development (mouse: Flach et al. 1982; cat: Hoffert et al. 1997).

Our aim was to determine the onset and pattern of embryonic gene transcription in early cat embryos beginning with the 2-cell stage. Assuming that the only resource of mRNA in the oocyte is of maternal origin, any increase in mRNA abundance in the whole embryo (independently of cleavage stage) in comparison to the mature oocyte would indicate an embryonic RNA transcription. Based on this assumption, we expressed relative mRNA amounts per embryo and not per single cell.

We clearly showed that in vitro cat embryos are able to transcribe from first cleavage onward. With the exception of IGF2R, the relative amount of mRNA increased during the development from matured oocytes to 2-cell embryos for all examined genes. These findings are not consistent with those of Hoffert et al. (1997), who detected first RNA generation in 5–8-cell cat embryos. In other species, especially bovine, the application of [3H]uridine labeling in more recent studies yielded different results compared with previous studies, suggesting improvements of this method during the past 15 years. In particular, older surveys found first gene activation in bovine embryos at the 8- or 8–16-cell stage (Camous et al. 1986, Kopecny 1989, Kopecny et al. 1989), whereas more recent studies detected the onset of transcription as early as in 2-cell embryos (Memili et al. 1998, Memili & First 2000). Therefore, a first wave of EGA is suggested to start immediately after fertilization followed by a second, major wave at a more advanced developmental stage. This was described for murine and bovine embryos (Memili & First 2000, Wrenzycki 2007). Apparently, the older [3H]uridine studies recognized the major activation, which coincides with the developmental block in vivo, suggesting that the transcriptional onset at the 5–8-cell stage found by Hoffert et al. corresponds MET, and we suspected dramatic increases in relative mRNA levels after reaching that certain stage in cat embryos.

In contrast to this expectation, we did not find a major activation around the 5–8-cell stage in any of our analyzed genes. Only POU5F1 showed its major activation during development from 8–16-cell embryo to morula. DNMT3A mRNA abundance multiplied dramatically during progress from morula to blastocyst. In contrast to that, DNMT1 mRNA levels increased constantly during the analyzed period, similar to those of ACTB. Owing to the methylation maintenance function of DNMT1 (Bestor 1992), this increase can be easily explained by the constant need for methylated cytosines during embryonic cleavage. DNMT3A establishes new methylation marks during cellular differentiation and is involved in de novo methylation processes in the early embryo (Okano et al. 1999). Therefore, DNMT3A expression pattern is supposed to precede the remethylation, which occurs at morulae in mice and after the 8-cell stage in cattle (Oswald et al. 2000, Dean et al. 2001). Ovine and rabbit embryos, however, do not show a stage-specific demethylation—remethylation (Beaujean et al. 2004, Young & Beaujean 2004). Thus, in these species, both DNMTs must be present equally during embryogenesis. The demethylation—remethylation pattern is still unknown for the cat embryo. The very early expression we found for DNMT3A might indicate a very early start of remethylation in the cat embryo, whereas an expression increase from morula to blastocyst might reflect the stem cell differentiation from toti- to pluripotency.

The characterization of the activation of GJA1 as well as IGF1R is difficult. There seemed to be a major onset of GJA1 expression during progress from 8–16-cell embryo to morula similar to that observed in POU5F1; however, GJA1 levels decreased from morula to blastocyst stage. Studies on bovine in vitro embryos confirm our pattern.

Figure 3 Relative mRNA abundance in morulae of different origins. IVF_fr, IVF with fresh sperm; IVF_cr, IVF with cryopreserved sperm; ICSI_fr, ICSI with fresh sperm; ICSI_cr, ICSI with cryopreserved sperm. Values shown as mean ± s.e.m.
for GJA1, whereas in vivo blastocysts showed increasing GJA1 mRNA levels (Wrenzycki et al. 1996, Lonergan et al. 2003). In contrast to Lonergan et al. (2003), who observed a decrease in IGF1R mRNA between zygote and 8-cell embryo and an increase afterwards until the blastocyst stage in in vitro cattle embryos, IGF1R mRNA abundances in our study showed no considerable alteration during the whole period. The IGF1R expression in cats could also be affected by maturation medium supplementation (FBS/BSA/PVP) as described by Warzych et al. (2007) for cattle embryos.

IGF2R was the only examined gene in domestic cat embryos whose mRNA level did not increase from the two-cell stage onward. It was activated (transcribed) after reaching the blastocyst stage and might therefore play a role in cell differentiation.

Based on the obtained expression pattern of genes in early cat embryogenesis, we conclude that the remarkable differences in the expression indicate a differential need for specific transcripts during each embryonic stage, as already shown for housekeeping and functional genes in cattle (Bilodeau-Goeseels & Schultz 1997). Monitoring single gene expression pattern by RT-PCR will not necessarily reflect the overall MET, which is defined as the stage when embryonic development stops because of transcription inhibition in the embryo (Memili et al. 1998, Memili & First 2000).

Our second aim was to compare in vitro produced cat morulae by their mRNA expression. In other species, early gene expression analysis was successfully applied to characterize the impact of embryo production methods (in vivo, in vitro, cloning, and parthenogenesis) on embryo quality (Corcoran et al. 2006, Wrenzycki et al. 2006, Nowak-Imialek et al. 2008). According to Hoffert et al. (1997), the EGA in cat embryos starts at 5–8-cell stage and the morula seems to be a critical phase in feline embryonic development. Under the condition of transcription inhibition with α-amanitin, embryonic development stops just before morula stage (Hoffert et al. 1997) and development under suboptimal in vitro conditions will also not go beyond morulae (Herrick et al. 2007). Therefore, we hypothesized that any influence of assisted reproductive techniques on the pattern of EGA must be expressed most profoundly in the morula stage.

Clarifying and controlling the effect of sperm source, fertilization, and culture technique in in vitro gamete handling is a general necessity. The application of techniques such as ICSI for the treatment of male infertility demands increasing attention to possible influences of sperm quality and source on the zygote’s and the embryo’s development (Gao et al. 2006). Suggestions that ICSI may result in impaired blastocyst development were not confirmed (Terriou et al. 1995, Staessen et al. 1999, Van Landuyt et al. 2005). Similarly, within our experiments no differences were observed in the rate and timing of embryonic development between the different fertilization methods and sperm sources used.

So far, the influence of the sperm source in gene expression studies has been ignored, although there is increasing evidence that the sperm quality also has a strong influence on embryo development (Loutradi et al. 2006, Menezo 2006). Sperm cell defects, such as teratozoospermia (De Vos et al. 2003, Junca et al. 2009) and chromosomal abnormalities, inhibit early embryo development (Shoukir et al. 1998, Nasr-Esfahani et al. 2005, Menezo 2006). In rats, reduced germ cell quality induced by exposure of males to anticancer drugs or lead was accompanied by defective embryonic development and deregulated EGA (Gandley et al. 1999, Hales et al. 2005). In cats, a correlation between poor centrosomal function and embryo outcome after ICSI was demonstrated (Comizzoli et al. 2006b).

Our results on relative mRNA levels of certain genes in morulae might indicate that embryonic gene expression is affected by the sperm source (fresh or frozen–thawed spermatozoa) and the method of fertilization (in vitro versus ICSI). By trend, the highest specific relative mRNA abundance was found in morulae produced by IVF with fresh sperm. This might suggest that this group is composed of the best quality embryos. The strongest differences to that group were found in ICSI morulae produced with cryopreserved sperm, followed by ICSI embryos fertilized with fresh sperm and IVF with previously frozen sperm.

To finally verify the effect of sperm source and fertilization method on EGA in cats, more embryos per group and in vivo produced embryos as physiological standards are needed. As domestic cats have no economic value like livestock species, where artificial reproduction methods are well established and applied, in vivo cat embryos are not easy to obtain in adequate numbers. Therefore, we were not able to compare the embryos derived in our study by different in vitro techniques with in vivo embryos, but only between each other. We assume that embryos produced by IVF with fresh spermatozoa are the least artificial embryos within our examinations and therefore are closest to in vivo embryos, but still differences between in vitro and in vivo produced embryos must be considered. This had already been demonstrated in other species (cattle: Corcoran et al. 2006; Lonergan et al. 2003; pig: Magnani & Cabot 2008). Finally, it would be of great importance to verify any kind of effects by transferring in vitro produced embryos into recipients.

Future studies should focus on the establishment of in vitro approaches which are as similar to the in vivo conditions as possible to enhance embryo survival and healthy development. Embryo morphology and blastocyst rate are not reliable parameters to identify deregulation in EGA and MET that is correlated to severe abnormalities such as increased gestation length, birth weight, or fetal losses as shown for cloned and in vitro
produced embryos (Hasler et al. 1995, Kruip & denDaas 1997, Niemann & Wrenzycki 2000). Thus, molecular markers will play a key role in the quality assessment of in vitro techniques and in our understanding of gene transcription during embryonic development.

Materials and Methods

Sequence analysis and primer design
Apart from a WGS sequence (accession AANG00000000), only few and short felid sequences are available from public databases. Therefore, primers for the genes of interest were designed based on alignments of highly conserved nonfelid species sequences (Table 1) using CLC sequence viewer 5 (www.clcbio.com). Genes included in our analysis were DNMT1, DNMT3A, GJA1, IGF1R, IGF2R, and POU5F1. Obtained PCR products were sequenced (SMB GmbH, Berlin, Germany) and aligned against cat WGS. When necessary, specific nesting primers were designed. Primers for β-actin (ACTB) were designed directly from an available cat sequence (Table 1). For rabbit globin amplification, primers designed by Wrenzycki et al. (2001) were used. All primers were obtained from Biotez Berlin-Buch GmbH (Berlin, Germany; Table 1).

In vitro production of cat embryos
All chemicals were purchased from Sigma–Aldrich unless stated otherwise and of the highest purity available.

Ovaries and testes were randomly obtained from either free-ranging or indoor cats ovarioctomized or castrated by local veterinary clinics. Both ovaries and testes were stored in 50 ml Greiner tubes at 4 °C and shipped in styrofoam boxes. Ovaries were kept in Minimum Essential Medium Eagle HEPES Modification supplemented with 3 mg BSA/ml and 1× Antibiotic Antimycotic Solution (A5955). Testes were maintained without medium. Samples arrived at the laboratory within at least 4 h after surgery. Ovaries were processed immediately whereas testes were stored for 24 h at 4 °C.

Ovaries were sliced in washing medium consisting of Medium 199 containing Earle’s salts, supplemented with 3 mg BSA/ml, 0.1 mg cysteine/ml, 1.4 mg HEPES/ml, 0.25 mg sodium pyruvate/ml, 0.6 mg sodium lactate/ml, 0.15 mg l-glutamine/ml, and 0.055 mg gentamicin/ml. Collection and quality assessment of cumulus oocyte complexes were performed under a stereomicroscope. High-quality oocytes (homogenous dark cytoplasm, intact cumulus layers) were placed into 400 μl of in vitro maturation medium (washing medium supplemented with 0.02 IU of FSH and LH/ml) under 400 μl mineral oil. Maturation proceeded at 38.5 °C and 5% CO2 in a humidified air atmosphere for 24 h.

Sperm cells were isolated by mincing the cauda epididymis in 1 ml TALP (Tyrode’s salts solution supplemented with 6 mg BSA/ml, 1.2 mg HEPES/ml, 1.1 mg sodium lactate/ml, 0.15 mg l-glutamine/ml, and 0.1 mg sodium pyruvate/ml). After motility assessment, the cell suspension was centrifuged at 500 g for 5 min and the pellet resuspended in 100 μl of medium. Sperm cell concentration was determined in a Neubauer counting chamber.

For cryopreservation, the sperm solution was diluted 1:3 in TEST buffer (20 mM TES, 10 mM Tris, 1 mM fructose, and 15% egg yolk) with 7.5% glycerol (v/v) according to Schmehl et al. (1986) to a final concentration of 20×10⁶ motile spermatozoa/ml. Cryo tubes (Greiner Bio-One, Frickenhausen, Germany) were prepared with 300 μl sperm solution each and equilibrated for at least 2 h at 4 °C in a double water bath. Sperm cells were frozen in a Nicool Freezer (Minitüb, Tiefenbach, Germany) according to Lengwinat & Blottner (1994) with cooling rates of −1 °C/min to −25 °C and −30 °C/min to −100 °C and then plunged into liquid nitrogen. Seeding occurred at −7 °C. For IVF, sperm was thawed in a water bath at 38 °C, washed in TALP, centrifuged for 5 min at 500 g, and resuspended in TALP.

IVF was performed after 24 h of in vitro maturation in 400 μl of TALP medium supplemented with 2.2 IU heparin at 38.5 °C and 5% CO2 in an air atmosphere for 16–18 h. The final sperm concentration in the fertilization drop was 1×10⁵ motile sperm/ml and 5×10³ motile spermatozoa/ml for fresh and frozen–thawed spermatozoa respectively.

For ICSI, oocytes were examined for the presence of a clear identifiable polar body after removal of cumulus cells by incubation in 300 μg hyaluronidase/ml (5 min). Sperm solution was diluted 1:10 in 10% PVP medium (Gynemed GmbH, Lehnssan, Germany). Single motile sperm were immobilized by drawing the injection pipette (5 μm inner diameter, Gynemed GmbH) across the midpiece. Then, sperm were pulled into the pipette and injected head first from the 3 o’clock position while the polar body was at the 6 or 12 o’clock position. The ICSI procedure was performed at 38.5 °C under an inverted microscope (Axiovert 100, Carl Zeiss, Jena, Germany).

ICSI is known for its potential to activate cat oocytes even without any sperm injection (Bogliolo et al. 2001, Comizzoli et al. 2006a). To prove that our ICSI-derived embryos are not parthenogenetically activated embryos, we performed sham injection as a negative control. The procedure was the same described for normal ICSI, but without injection of a sperm cell. In addition, we activated sham-injected oocytes by incubation in 7% ethanol for 5 min. After ICSI–sham injection of a total of 40 oocytes (in seven experiments) and alcohol activation, no cleavage was obtained. This was in contrast to previously published data on parthenogenetic activation by sham injection (Bogliolo et al. 2001, Comizzoli et al. 2006a). We can only speculate about that effect, though differences in culture systems, transportation conditions, ICSI procedure, and cat populations are all potential sources for the discrepancy. According to our parthenogenetic controls, we concluded that all embryos produced in the current effort by IVF or ICSI were ‘real’ and viable.

Parthenogenetic activation in terms of spontaneous cleavage during the in vitro maturation without any further treatment was observed very rarely (5%, 15 of 300 oocytes). Nevertheless, we were able to obtain one pool of parthenogenetic morulae (n=2), which was used as parthenogenetic reference for gene expression study, although it could not be included into statistical analyses.

Embryo culture was performed in embryo culture medium consisting of M16 Medium supplemented with 0.03 mg gentamicin/ml and 1× MEM nonessential amino acids at 38.5 °C in air with 5% CO2. After 50–60 h post insemination, embryos
were placed into embryo culture medium supplemented with 10% FCS (v/v). The progress of embryonic development was monitored daily and normal speed embryos were collected for RNA isolation (2-cell embryos at day 1; 4-cell embryos at day 2; 8–16-cell embryos at day 3; morulae at day 4 or 5; blastocysts at days 6–8). Owing to the dark cytoplasm of cat oocytes, a differentiation between zygotes and unfertilized oocytes is not possible; therefore, pronuclear stages could not be collected for RNA extraction. Before that, all residual cumulus cells were removed by aspirating each embryo into a narrow pipette. The success of the removal was carefully controlled using an inverted microscope.

In addition, oocytes before and after maturation were obtained and freed of cumulus cells by 300 μg hyaluronidase/ml (5 min). Parthenogenetic embryos (morulae) were obtained by culturing unfertilized mature oocytes for 4–5 days.

All oocytes and embryos were washed at least three times in 20 μl droplets of Dulbecco’s PBS and then frozen in a minimum of PBS at −80 °C.

The average maturation rate was 60.4±4.6% (n=689). Cleavage rate after IVF was lower (26.0±5.9%, n=416) than after ICSI (43.6±4.5%, n=446). Development until blastocyst stage (after IVF) occurred with a percentage of about 25% (n=88).

**Determination of relative mRNA abundance by RT-PCR**

Total RNA was isolated from each pool (see experimental design described below) of embryos with RNeasy Micro kit (Qiagen) following the manufacturers’ instructions (RNeasy Micro Handbook, second edition, December 2007). Samples were stored at −80 °C and transferred onto ice shortly before RNA preparation. Briefly, embryonic cells were lysed and homogenized in the presence of 4 μg poly-A carrier RNA and 1 μg rabbit globin RNA. The nonfelid globin RNA served as an internal control to assess the efficiency of the RNA isolation process and RT-PCR as well as for calculation of relative mRNA abundances. In many gene expression studies, ACTB is used as a control gene, because its ‘housekeeping’ function is suggested to be not regulated. However, within the early cat embryo, the RNA expression of ACTB was found to be heavily dependent on the developmental stage making its application as an internal control unreliable. Therefore, we introduced the nonfelid standard (rabbit globin) to calculate the relative RNA abundance. This approach was also used in several studies on bovine embryos (Wrenzycki et al., 1999, 2001, 2006). After adding 70% ethanol, total RNA was bound to the MinElute spin column by centrifugation (Universal 320R, Hettich, Tuttingen, Germany) followed by DNA digestion with DNase1. After washing with buffer and 80% ethanol, total RNA was eluted in 14 μl RNAse-free water and stored at −80 °C until RT.

Synthesis of single-strand cDNA was carried out with the RevertAid First, Strand cDNA Synthesis kit (Fermentas GmbH, St Leon-Rot, Germany) in a total volume of 40 μl according to the manufacturer’s description. In brief, total embryonic RNA and 1 μg Oligo-dT(18) primer and 25 μl H2O DEPC were incubated at 65 °C for 5 min, then quickly transferred onto ice. After addition of 1 X reaction buffer, 1 U RiboLock RNAse inhibitor, 1 mM dNTP Mix, and 5 U RevertAid M-MulV Reverse Transcriptase, incubation (42 °C, 60 min) was performed followed by 5 min of enzyme inactivation at 70 °C within a block thermostat. Resulting cDNA was stored at −20 °C.

DNA was amplified using a G-Storm GS1 Thermocycler (Gene Technologies Ltd, Essex, UK) in a total volume of 25 μl using the Fast Start Taq Polymerase dNTP Pack (Roche Diagnostics GmbH). The reactions occurred in 1 X PCR buffer (50 mM Tris/ HCl, 10 mM KCl, 5 mM (NH4)2SO4, and 2 mM MgCl2), 200 μM of each dNTP, 0.8 μM sequence-specific primer, and 1 IU Taq Polymerase. Six minutes of initial denaturation at 95 °C followed 40 cycles of 30 s denaturation at 95 °C, 30 s annealing at 54.6 °C (DNMTI), 56.2 °C (DNMT3A, GJA1, POUSFI, IGF1R, and IGF2R), or 59.8 °C (ACTB, rabbit globin) and 30 s of elongation at 72 °C. After final elongation at 72 °C for 7 min PCR products were stored at −20 °C. Amplified specific PCR products were analyzed on a 1.2% agarose gel (Peqlab Biotechnology GmbH, Erlangen, Germany) in 1 X TBE (90 mM Tris base, 90 mM boric acid, and 2 mM EDTA) containing 0.25× Gelred Nucleic Acid Stain (Biotrend Chemikalien GmbH, Köln, Germany). A 50 bp DNA ladder was used as a size marker (Fermentas GmbH).

Signal intensities were measured on a u.v transilluminator and determined with ImageJ (National Institutes of Health, Bethesda, Maryland, USA, http://rsweb.nih.gov/ij/). The relative amounts of the mRNAs of interest were assessed by dividing the specific fragment gel band intensity of each template by the intensity of the corresponding globin band. Subsequently, relative mRNA abundances measured for the pools were divided by the number of oocytes or embryos. For statistical analysis of the influence of fertilization method and sperm source on relative mRNA expression, a two-factor ANOVA was performed.

**Examination of the temporal pattern of relative mRNA abundance**

For the examination of the temporal pattern of mRNA abundance in in vitro preimplantation felid embryos, germinal vesicle and metaphase II oocytes as well as different embryonic cleavage stages were collected. We pooled oocytes and embryos so that in each tube approximately the same amount of RNA was used.

**Figure 4** Representative agarose gels of a semiquantitative RT-PCR analysis of DNA methyltransferase 1 in different embryonic developmental stages. (A) Relative mRNA abundance in single germinal vesicle oocytes (CV), metaphase II oocytes (MII), 2-cell embryos (2-cell), 4-cell embryos (4-cell), 8–16-cell embryos (8–16-cell), morulae, and blastocysts; 50 bpl, 50 bps DNA ladder; ntc, no template control. (B) Relative mRNA abundance in 40 germinal vesicle oocytes (CV), 40 metaphase II oocytes (MII), 20 2-cell embryos (2-cell), 10 4-cell embryos (4-cell), 5 8–16-cell embryos (8–16-cell), 2 morulae, and 1 single blastocyst; 50 bpl, 50 bps DNA ladder; ntc, no template control.
present before performing RNA extraction and RT. RNA extraction as well as cDNA production from a single oocyte or early embryo is ineffective and results in gel signals that are hardly measurable (Fig. 4). Thus, 40 immature and 40 matured oocytes (both free from cumulus cells), 20 2-cell embryos, 10 4-cell embryos, 5 8–16-cell embryos (two replicates), and 2 morulae (six replicates) were pooled. Furthermore, single blastocysts (seven replicates) were used for RNA isolation.

**Comparison of the relative mRNA amounts in embryos of different origins**

To evaluate the influence of fertilization method as well as sperm source, relative mRNA expression levels in embryos of different origins were measured and compared. Pools of two 4–5-days-old morulae derived by i) IVF with fresh sperm (six replicates), ii) IVF with frozen–thawed sperm (six replicates), iii) ICSI with fresh sperm (four replicates), and iv) ICSI with frozen–thawed sperm (three replicates) were collected. In addition, one pool spontaneously cleaved (parthenogenetic) morulae could be obtained for relative mRNA evaluation.

**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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**References**


