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THE INFLUENCE OF THE OOPLASM ON DNMT1 AND DNMT3A GENE EXPRESSION

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ABSTRACT

Developmental incompetence of embryos constructed by somatic cell nuclear transfer is caused by insufficient reprogramming of the transferred nucleus to a state equivalent to that of an early embryonic nucleus. Preceding studies have shown that the transcription of DNA methyltransferase (DNMT) genes in mammalian oocytes and preimplantation embryos is a species-dependent process and the incomplete DNA methylation correlates with the nuclear transfer failure rate in mammals. In the present study the transcription of DNMT1 and DNMT3a genes in early embryonic stages of intergeneric nuclear transfer (iSCNT) embryos (bovine, porcine) was detected by RT-PCR. Based on the diverse timing of major genome activation during embryonic development in bovine and porcine embryos, the strong influence of the ooplasm on transferred somatic cell nucleus was expected. Despite the presence of DNMT1 and DNMT3a mRNA of maternal origin, expression of somatic DNMT1 and DNMT3a genes was not detected and the development of intergeneric embryos stopped at 4-cell stage. These results indicate that the species-specific epigenetic reprogramming during early embryogenesis is strongly influenced by ooplasm environment.

Keywords: bovine embryos; porcine embryos; intergeneric nuclear transfer; DNMT1 and DMT3a gene expression

INTRODUCTION

During embryonic development different cells and tissues gain different programs of gene expression. It is thought that this is mainly regulated by epigenetic modifications such as DNA methylation, histone tail modifications and non-histone proteins that bind to the chromatin (Bird, 2002; Li, 2002). For most cell types in the body, these epigenetic marks become fixed when the cells differentiate or exit the cell cycle. In normal developmental situations, some cells may undergo major epigenetic reprogramming, involving the removal of epigenetic marks in the nucleus, followed by establishment of a different set of marks (Rideaut *et al.*, 2001).

Epigenetic modifications occur during the life cycle in two phases: during gametogenesis and preimplantation development. Primordial germ cells (PGCs) originate from somatic tissue and develop into mature gametes over an extended period of time (Morgan *et al.*, 2005). Their genome undergoes DNA demethylation in the embryo between E11.5 and E12.5, including imprinted genes. Following demethylation, the genomes of the gametes are *de novo* methylated and acquire imprints; this process continues up to E18.5 in males and in maturing oocytes before ovulation in

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females (Saitou *et al.*, 2012). After the fertilization the next round of reprogramming begins and lasts during the early embryonic development. The paternal genome actively decreases its methylation level and its histones absent some modifications of polypeptide tails in comparison to histones in the maternal pronucleus. The embryo genome is passively demethylated during early cell cycles before blastulation. Despite this methylation loss, imprinted genes maintain their methylation through this preimplantation reprogramming. *De novo* methylation roughly coincides with the differentiation of the first two lineages of the blastocyst stage, and the inner cell mass (ICM) is hypermethylated in comparison to the trophectoderm (TE). These early lineages set up the DNA methylation status of their somatic and placental derivatives. Histone modifications may also reflect this DNA methylation asymmetry. Particular classes of sequences may not conform to the general genomic pattern of reprogramming shown (Mann *et al.*, 2003).

DNA methylation critically depends on the activity of specific enzymes, the DNA methyltransferases (DNMTs). Five mammalian cytosine DNA methyltransferases have been identified to date (Bestor, 2000). DNA-methyltransferase 1 (DNMT1) is a maintenance enzyme that is responsible for restoring methylation of hemi-methylated CpG dinucleotides after DNA replication (Bestor, 1992). An oocyte-specific form, DNMT1o, is present at high concentrations in mature oocytes and lasts during early zygotic stages. Gene targeting experiments suggest that DNMT1o has a role in maintaining methylation marks at maternally imprinted genes in mice (Howell *et al.*, 2001).

Additional types of the vertebrate cytosine methyltransferase are DNMT3a and DNMT3b. These enzymes catalyze *de novo* methylation and are thus essential for establishing DNA methylation during development (Okano *et al.*, 1998).

The aim of this study was to detect the expression of DNMT1 and DNTM3a genes at different stages of embryonic development of bovine vs. porcine intergeneric nuclear transfer (iSCNT) embryos and compare these results with expression in parthenogenetically activated porcine and bovine oocytes. The influence of different ooplasm environment on DNMT1 and DNMT3a genes expression was expected.

MATERIAL AND METHODS

Oocyte recovery and *in vitro* maturation

Bovine cumulus-oocyte complexes (COCs) were isolated by ovarian slicing from slaughtered cattle of different origin. Selected COCs were matured *in vitro* in tissue culture medium 199 (TCM 199; Sigma-Aldrich, Germany) containing L-glutamine and 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Sigma, St. Louis, MO, USA) supplemented with 22 mg mL^{-1} pyruvate, 2.2 mg mL^{-1} $NaHCO_3$, 50 mg mL^{-1} gentamicin, 10 IU mL^{-1} eCG, and 5 IU mL^{-1} of hCG (Suigonan, Intervet, Tönisvorst, Germany). Oocytes were *in vitro* matured in 100 ml droplets of maturation medium under silicone oil for 19 h at 39 °C and 5 % CO_2 in humidified atmosphere. After maturation, cumulus cells were completely removed by vortexing with 0.1 % hyaluronidase in phosphate-buffered saline (PBS). MII oocytes were chosen for further experiments.

Ovaries from pre-pubertal gilts were collected from local abattoirs. Oocytes with at least three complete layers of cumulus cells were collected and matured *in vitro* for 40 h (Holker *et al.*, 2005). Then, matured oocytes were transferred to TL-HEPES supplemented with 0.1 % hyaluronidase and incubated for 5 min. Cumulus cells were removed by repeated pipetting. Denuded oocytes were transferred to Tyrode's lactate HEPES (TL HEPES) (Sigma, St. Louis, MO, USA) covered with mineral oil (Silicone fluid DC200, Serva Biochemica, Heidelberg).

iSCNT and parthenogenetic activation

For production of nuclear transfer embryos the MII oocytes were placed into TCM 199 medium enriched with gentamicin, Na-pyruvat, $NaHCO_3$ and BSA (TCM-air) containing 5 mg mL^{-1} Hoechst 33342 and 7.5 mg mL^{-1} cytochalasin B for 8 min.

The oocytes were enucleated by aspirating the first polar body and the MII plate. A single fibroblast was transferred into the perivitelline space of the recipient enucleated oocyte. Oocyte-fibroblast cell couplets were electrically fused in a 0.285 mM mannitol-based medium containing 0.1 mM $MgSO_4$ and 0.05 bovine serum albumin (BSA) with Multipolator® machine (Eppendorf AG, Germany). Fused cell hybrids (iSCNT) and intact oocytes (parthenogenetic activation) were chemically activated by 5 µM ionomycin. After activation, the embryos were washed and cultured in 30 ml droplets of synthetic oviduct fluid medium supplemented with amino acids and BSA (SOFaa; Sigma-Aldrich, Germany) supplemented with 0.4 % BSA at 39 ºC in 5 % O_2, 5 % CO_2 and 90 % N_2 in modular incubation chambers. The embryos were collected at 2-, 4-, 8- and 16-cell stages for further processing.

RNA extraction and RT-PCR

Total RNA was extracted from pools (triplicates) of 10 embryos each at 2-cell, 4-cell, 8-cell and 16-cell stages from iSCNT/parthenogenetic embryos using Dynabeads® mRNA DIRECT™ kit (Life Technologies, USA). Subsequently, RT was carried out in total volume of 20 µl using 2.5 µM random hexamers. The species-specific primers for DNMT1 and DNMT3a genes were designed for determination of *de novo* synthesis

Table 1: Species specific primer sequences designed for detection of DNMT1 and DNMT3a genes expression in bovine and porcine embryos

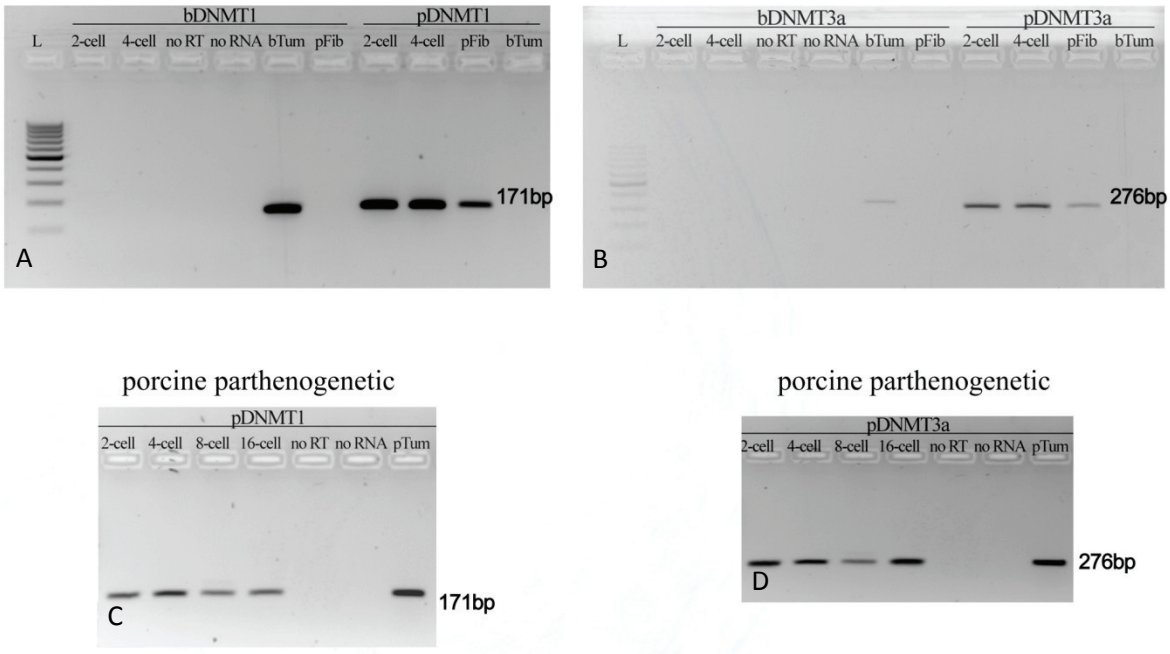
Gene	Sequence (5´-3´)	Length
bDNMT1	**F**-ACCGAGTGCTTGCAGTACCT **R**-GCTGAGGCAAATCCTCGTAA	154 bp
bDNMT3a	**F**-CAAAGCAGCTGACGATGAAC **R**-GCAGGACCTCGTAGATAGCC	296 bp
pDNMT1	**F**-AGTGCGTTCAGTGTGGACAG **R**-CGGTCAGTTTGTGTTGGAGA	171 bp
pDNMT3a	**F**-CAGTACGACGATGACGGCTA **R**-GTCAAATTCCTGGTCGTGGT	276 bp

b – bovine specific, p – porcine specific

of epigenetic enzymes (Tab. 1). As positive controls, porcine and bovine parthenogenetic embryos were used.

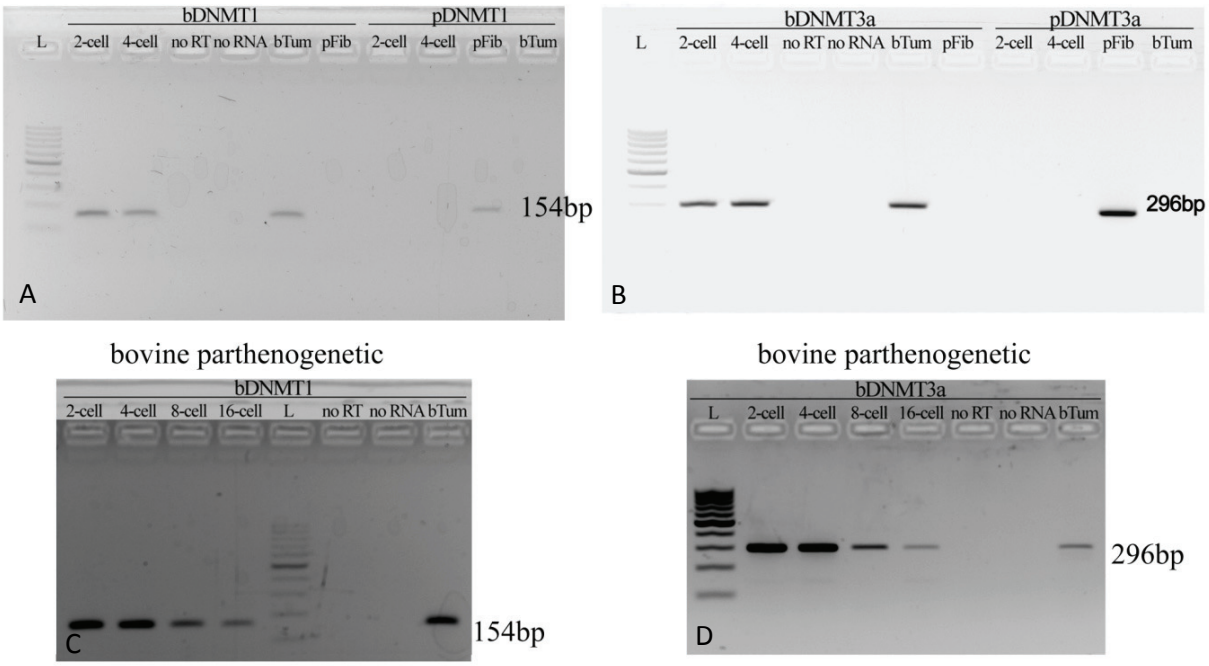
RESULTS AND DISCUSSION

All the pools were done in triplicate and contained iSCNT embryos (n = 10). The species-specific primers for DNMT1 and DNMT3a genes were designed for determination of *de novo* synthesis of epigenetic enzymes. Gene transcription for bovine DNMT1 (bDNMT1) and DNMT3a (bDNMT3a) was not observed in 2- and 4-cell stage embryos generated by bovine fibroblast transfer into the porcine ooplasm, however, positive results were obtained by using primers for pig DNMT1 (pDNMT1) and DNMT3a (pDNMT3a) (Fig. 1A, B). As positive



bTum – bovine tumour cells (control of primer specificity), pFib – porcine fibroblasts (control of primer specificity), pTum – porcine tumour cells (control of primer specificity), noRT – no reverse transcriptase in mastermix (negative PCR control), no RNA – no RNA in mastermix (negative PCR control), L – 100 bp DNA ladder (Abnova, Germany)

Fig. 1: (A-B) bovine DNMT1 (bDNMT1) (A) and bovine DNMT3a (bDNMT3a) (B) gene expression in comparison with porcine DNMT1 (pDNMT1) (A) and porcine DNMT3a (pDNMT3a) (B) in iSCNT embryos constructed by injecting the bovine fibroblast into the porcine oocyte indicates the lack of *de novo* transcription of bovine-specific epigenetic enzymes. (C-D) porcine DNMT1 (pDNMT1) (C) and porcine DNMT3a (pDNMT3a) (D) gene expression in porcine embryos after parthenogenetic activation indicates the presence of porcine-specific transcripts of epigenetic enzymes from 2-cell to 16-cell stage



bTum – bovine tumour cells (control of primer specificity), pFib – porcine fibroblasts (control of primer specificity), noRT – no reverse transcriptase in mastermix (negative PCR control), no RNA – no RNA in mastermix (negative PCR control), L – 100 bp DNA ladder (Abnova, Germany)

Fig. 2: (A-B) bovine DNMT1 (bDNMT1) (A) and bovine DNMT3a (bDNMT3a) (B) gene expression in comparison with pig DNMT1 (pDNMT1) (A) and pig DNMT3a (pDNMT3a) (B) in iSCNT embryos constructed by injecting the porcine fibroblast into the bovine oocyte indicates the lack of *de novo* transcription of porcine-specific epigenetic enzymes. (C-D) bovine DNMT1 (pDNMT1) (C) and bovine DNMT3a (pDNMT3a) (D) gene expression in bovine embryos after parthenogenetic activation indicates the presence of bovine-specific transcripts of epigenetic enzymes from 2-cell to 16-cell stage

controls, porcine parthenogenetic embryos were used (Fig. 1C, D).

In the 2- and 4-cell stage embryos constructed using porcine fibroblast and bovine ooplasm only the bovine specific primers (bDNMT1, bDNMT3a) showed positive signals (Fig. 2A, B). Based on the different timing of major genome activation during the embryonic development in bovine and porcine embryos, the strong influence of ooplasm on introduced fibroblast was expected. Despite the mRNA presence of DNMT1 and DNMT3a enzyme of the oocyte origin, *de novo* transcription of somatic DNMT1 and DNMT3a genes was not detected and iSCNT embryos did not develop beyond the 4-cell stage. In parthenogenetic embryos, the continuous supplementation of epigenetic enzyme transcripts is maintained by the major genome activation including the expression of DNMT1 and DNMT3a genes (Fig. 2C, Fig. 2D).

DNA methyltransferase 1 (DNMT1) is very intensively studied DNA methyltransferase and is thought to be responsible for maintaining the methylation patterns following the DNA replication (Dean *et al.*, 1998). DNMT3a and DNMT3b are responsible for *de novo* DNA methylation (Okano *et al.*, 1999). Previous studies have shown species-dependent expression patterns of DNMT genes in mammalian oocytes and preimplantation embryos (Vassena *et al.*, 2005) and also a correlation between incomplete DNA methylation and the lack of NT success in mammals (Dean *et al.*, 2001; Bortvin *et al.*, 2003). DNMT1 and DNMT3a mRNA in cattle were detected continuously from the 2-cell stage to the blastocyst stage produced *in vitro* (Golding and Westhusin, 2003), but the relative abundance of the Dnmt1 transcript significantly varied between *in vivo* and *in vitro* produced bovine embryos, with *in vivo* produced embryos expressing significantly less DNMT1 (Wrenzycki *et al.*, 2001). Similarly, a significant increase in DNMT1 expression at the 8-cell stage was reported in porcine fibrillar sphere NT embryos compared with either *in vivo* produced or fetal porcine skin originated sphere

stem cell NT embryos (Zhu *et al.*, 2004). Comparing IVF and *in vivo* derived embryos, the relative abundance of DNMT1 transcript was significantly increased in NT embryos and only a low level of DNMT1 transcription was found for *in vivo* derived embryos (Kumar *et al.*, 2007). DNMT3a mRNA was expressed at all stages analyzed with a significant increase in abundance between the 8-cell and morula to blastocyst stages which coincides with initiation of cell differentiation processes.

Intergeneric SCNT has been considered as a very effective technique for studying the influence of the ooplasm on epigenetic reprogramming of introduced genome which subsequently leads to different aberrations during the embryogenesis. Therefore, the production of live offspring from intergeneric embryos has not been reported, and embryonic development beyond the stage of major embryonic genome activation is significantly hindered (Østrup *et al.*, 2011). Remodeling and reprogramming of the transferred genome is essential for successful embryonic development following SCNT.

The presented study is focused on the influence of different ooplasms (porcine and bovine) on the activation of *de novo* synthesis of DNMT1 and DNMT3a mRNA. It is already known that the matured mammalian oocytes are equipped with efficient amount of epigenetic enzymes and their transcripts required for initial epigenetic processes during early embryogenesis (Vassena *et al.*, 2005). However, these enzymatic supplies are not sufficient for complete reprogramming of transferred nuclei during SCNT, what results in embryonic development breakdown. The low efficiency of NT seems to be related to the inability of a somatic nucleus to undergo the normal changes in methylation as indicated by increased levels of DNMT1 or to the lack of *de novo* methylation triggered by low DNMT3a expression (Kumar *et al.*, 2007). To distinguish the source of epigenetic enzymes (ooplasmic and *de novo* synthesized) intergeneric nuclear transfer (pig vs. bovine and *vice versa*) was applied in combination with species-specific RT-PCR primers detecting DNMT1 and DNMT3a transcription. Our results significantly show the incompetence of introduced somatic nucleus to initiate and establish the continual expression of observed genes in the environment of ooplasm of different origin. This malfunction has led to embryonic development breakdown at the 4-cell stage.

CONCLUSION

Our results strongly indicate species-dependent and maternally controlled regulation of epigenetic reprogramming during early embryogenesis and importance of epigenetic enzymes in proper embryonic development.

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