Genome reprogramming during the first cell cycle in *in vitro* produced porcine embryos

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ABSTRACT: Conflicting data still exist regarding the extent of paternal pronuclear DNA demethylation in one cell-stage mammalian embryos. Demethylation of paternal pronuclear DNA was observed in *in vivo* produced porcine zygotes, whereas *in vitro* produced embryos do not show any or only weak paternal genome demethylation. In our experiments, we have used and compared two *in vitro* techniques commonly used for *in vitro* embryo production (*in vitro* fertilization and intracytoplasmic sperm injection) and then we evaluated the extent of labelling in both these groups after 5-methylcytosine (5-MeC) or dimethyl H3/K9 labelling. We have found no differences in the methylation pattern between both those techniques used for the production of embryos. Moreover, we did not detect any demethylation of paternal DNA after 5-MeC labelling at all. Contrary to this, labelling with dimethyl H3/K9 antibodies showed differences in labelling intensity between maternal and paternal genomes in 42% of zygotes after *in vitro* fertilization and in 44% of zygotes after intracytoplasmic sperm injection. Our results indicate that *in vitro* matured pig oocytes exhibit rather inconsistent methylation patterns. This inconsistency probably resulted from insufficient cytoplasmic maturation of oocytes and to a lesser extent from the *in vitro* technique for embryo production.

Keywords: epigenetics; pig; ICSI; IVF; methylation

In mammals, soon after fertilization the sperm genome undergoes rapid and extensive chromatin remodelling where sperm protamines are replaced by histones from the oocyte cytoplasm (McLay and Clarke, 2003). During the process of protamine-histone replacement and chromatin structure reorganization the major epigenetic modifications also take place. Among them, the most extensively studied is the demethylation of paternal pronuclear DNA.

The mechanisms and patterns of epigenetic reprogramming during early development do not seem to be, however, conserved among mammalian species. The paternal genome is actively demethylated in the rat, mouse and monkey (Dean et al., 2001; Yang et al., 2007; Zaitseva et al., 2007). In zygotes of some other species, like sheep and rabbit, the persisting methylation of both genomes in the zygote has been reported (Beaujean et al., 2004; Shi et al., 2004), the bovine paternal DNA is demethylated only partially (Dean et al., 2001). Different methylation patterns were reported for pig, human and goat embryos (Beaujean et al., 2004; Fulka et al., 2004). Especially in the pig some authors reported the extensive demethylation of paternal pronuclei (Dean et al., 2001; Fulka et al., 2006), whilst others were unable to see any differences between pronuclei, i.e. both remained methylated (Jeong et al., 2007a; Park et al., 2007). However later on, it has been shown that even in rabbit zygotes the paternal

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chromosomes become significantly demethylated. This process occurs only at advanced pronuclear development stages (Lepikhov et al., 2008).

Only fragmentary or no information, however, exists regarding the methylation patterns in *in vitro* produced porcine embryos with two different but most commonly used approaches, i.e. conventional *in vitro* fertilization vs. ICSI (intracytoplasmic sperm injection). These *in vitro* produced embryos could possess some defects in the methylation patterns (Bonk et al., 2008) that will arise from the method used for their production. Moreover, we can also expect that these defects in *in vitro* produced embryos may be also caused by incomplete cytoplasmic maturation of oocytes (Gioia et al., 2005).

There are two main technique used for *in vitro* production of porcine zygotes – intracytoplasmic sperm injection (ICSI) and *in vitro* fertilization (IVF). The technique of *in vitro* fertilization in pigs faces problems associated with extensive polyspermy (Funahashi, 2003). On the other hand, in the case of intracytoplasmic sperm injection, the boar sperm heads do not often decondense and consequently no fully developed male pronuclei are formed (Kren et al., 2003).

In our experiments, we evaluated the methylation patterns in porcine zygotes produced by both, abovementioned, methods.

MATERIAL AND METHODS

All chemicals and reagents were purchased from Sigma-Aldrich (Prague, Czech Republic) unless stated otherwise.

Oocyte isolation and maturation

Pig ovaries were obtained from a local slaughterhouse from gilts at an unknown stage of the oestrous cycle and transported to the laboratory within 1 hour in Dulbecco's phosphate-buffered saline (Gibco, Invitrogen, Prague, Czech Republic) containing 0.01% polyvinyl alcohol at 30–35°C. Porcine oocytes were isolated by dissecting antral follicles of 4–6 mm diameter as described by Moor and Trounson (1977). The follicles were opened in HTF-HEPES medium (Cambrex Bio Science, Verviers, Belgium) supplemented with 2 mg/ml bovine serum albumin. Groups of 25 to 35 oocyte-cumulus-complexes were cultured in 500 µl bicarbonate-buffered medium199 supplemented with 4 mg/ml of growth proteins of bovine serum (GPBoS, Sevapharma, Prague, Czech Republic), 0.5 µg/ml FSH, 0.5 µg/ml LH, 40 µg/ml sodium pyruvate, 70 µg/ml L-cysteine and 50 µg/ml gentamicin (Gibco, Invitrogen, Prague, Czech Republic) covered with paraffin oil (Carl Roth, Karlsruhe, Germany).

Oocyte maturation was carried out for 44 to 46 hours in a humidified atmosphere of 5% CO_2 in air at 38.5°C. Oocytes were denuded after maturation by incubating them briefly in 0.01% hyaluronidase followed by pipetting through a small-bore pipette.

Sperm preparation and *in vitro* fertilization

The commercially distributed boar spermatozoa (stored maximally for up to 5 days at 17°C, Chovservis, Hradec Králové, Czech Republic, diluted in Solusem, AIM Worldwide) were centrifuged at 700 × g for 5 minutes. After centrifugation, the sperm pellet was loaded with a small volume of extended dilution medium onto a discontinuous gradient of 2 ml of 80% and 5 ml of 40% isotonic Percoll (Amersham Pharmacia Biotech, Uppsala, Sweden) prepared with PBS in a 15-ml centrifugation tube and centrifuged for 15 minutes at 700 × g. Then the spermatozoa were washed twice in PBS-PVA by centrifugation at 700 × g for 5 minutes.

The sperm pellet was resuspended in 1 ml of modified Tris-buffered medium (mTBM) and the suspension was then diluted in mTBM (113.1mM NaCl, 3mM KCl, 20mM Tris, 11mM glucose, 7.5mM $CaCl_2 \times 2H_20$, 5mM sodium pyruvate, supplemented with 2 mg/ml BSA). Before *in vitro* fertilization or intracytoplasmic sperm injection spermatozoa were preincubated in mTBM for 1 hour.

Fertilization was performed in 1 ml of mTBM in 4-well dishes (Nunc, Roskilde, Denmark). Groups of 25–40 oocytes were placed into each well. A portion of sperm suspension was added to obtain the final concentration of 1 250 spermatozoa per oocyte. Sperm and oocytes were coincubated for 3 hours in the humidified atmosphere of 5% CO_2 at 38.5°C in an incubator.

After 3 hours, oocytes were washed in porcine zygote medium (PZM-3) and then cultivated in PZM-3 supplemented with 3 mg/ml BSA (Yoshioka et al., 2002) for 20–24 hours, each group of ten oocytes

in a 20 µl drop of cultivation medium covered with paraffin oil (Carl Roth, Karlsruhe, Germany) in an incubator with the humidified atmosphere of 5% CO_2 at 38.5°C.

Intracytoplasmic sperm injection

Spermatozoa were prepared as described above with a minor modification. After centrifugation, the sperm pellet was resuspended in mTBM (1.9mM $CaCl_2$×2 H_20) and diluted in 0.5 volume of mTBM medium to obtain the final concentration of 2 × 10^6 cells/ml. Spermatozoa were preincubated for 1–1.5 hours before use.

Microinjection was performed under an inverted microscope (IX71, Olympus Europa GMBH) in drops of HTF-HEPES medium (Cambrex Bio Science, Verviers, Belgium). 3 µl of preincubated spermatozoa suspension was added to a 3 µl-drop of polyvinylpyrrolidone medium (10% PVP; COOK Australia, Queensland, Australia) on the bottom of a manipulation dish (final concentration 1 × 10^6 cells/ml and 5% PVP). Only single motile spermatozoa were immobilized by hitting the mid-piece with the injection pipette and then the sperm was aspirated into the pipette. Sperm injection was performed as described previously by (Katayama et al., 2005).

The injected oocytes were cultured in PZM-3 for 20–24 hours, as described above.

Evaluation of pronuclei

We used only non-dividing zygotes (one-cell stage) for fixation and further labelling.

Zygotes were incubated for 10 minutes in HTF-HEPES medium (Cambrex Bio Science, Verviers, Belgium) supplemented with cytochalasin B (7.5 µg/ml) and then centrifuged for 10 minutes at 9.000 rpm to induce the stratification of the cytoplasm and thus to facilitate the visualization of pronuclei. Zonae pellucidae were then dissolved in acid Tyrode solution and oocytes were then fixed in 4% paraformaldehyde for one hour and then incubated in 4% paraformaldehyde with 0.01% Triton X-100 overnight. The following antibodies were used for labelling: anti-dimethyl H3/K9 (Upstate, Charlottesville, VA, USA) and 5-MeC (Eurogentec, Seraign, Belgium), essentially as described by Fulka et al. (2008).

The evaluation of pronuclei after labelling was performed under a confocal microscope (Olympus BX61). Pictures were adjusted by AnalySIS (Soft Imaging System GMBH, Munster, Germany) and Adobe Photoshop CS2 software.

RESULTS

Under our conditions, approximately 95% of oocytes reached metaphase II stage with the first polar body extruded. For the conventional *in vitro* fertilization and intracytoplasmic sperm injection

Table 1. DNA methylation reprogramming patterns in pig zygotes after different *in vitro* techniques

Type of fertilization		5-MeC (%)	Dimethyl H3/K9 (%)
IVF	MP–/FP+	0	8 (42)
	MP–/FP–	0	3
	MP+/FP+	12 (100)	11 (58)
	number of successfully labelled zygotes	12	19
	total zygotes	12	22
ICSI	MP–/FP+	0	11 (44)
	MP–/FP–	0	32
	MP+/FP+	9 (100)	14 (56)
	number of successfully labelled zygotes	9	25
	total zygotes	9	57

MP – male pronucleus; FP – female pronucleus; + positive labelling; – negative labelling

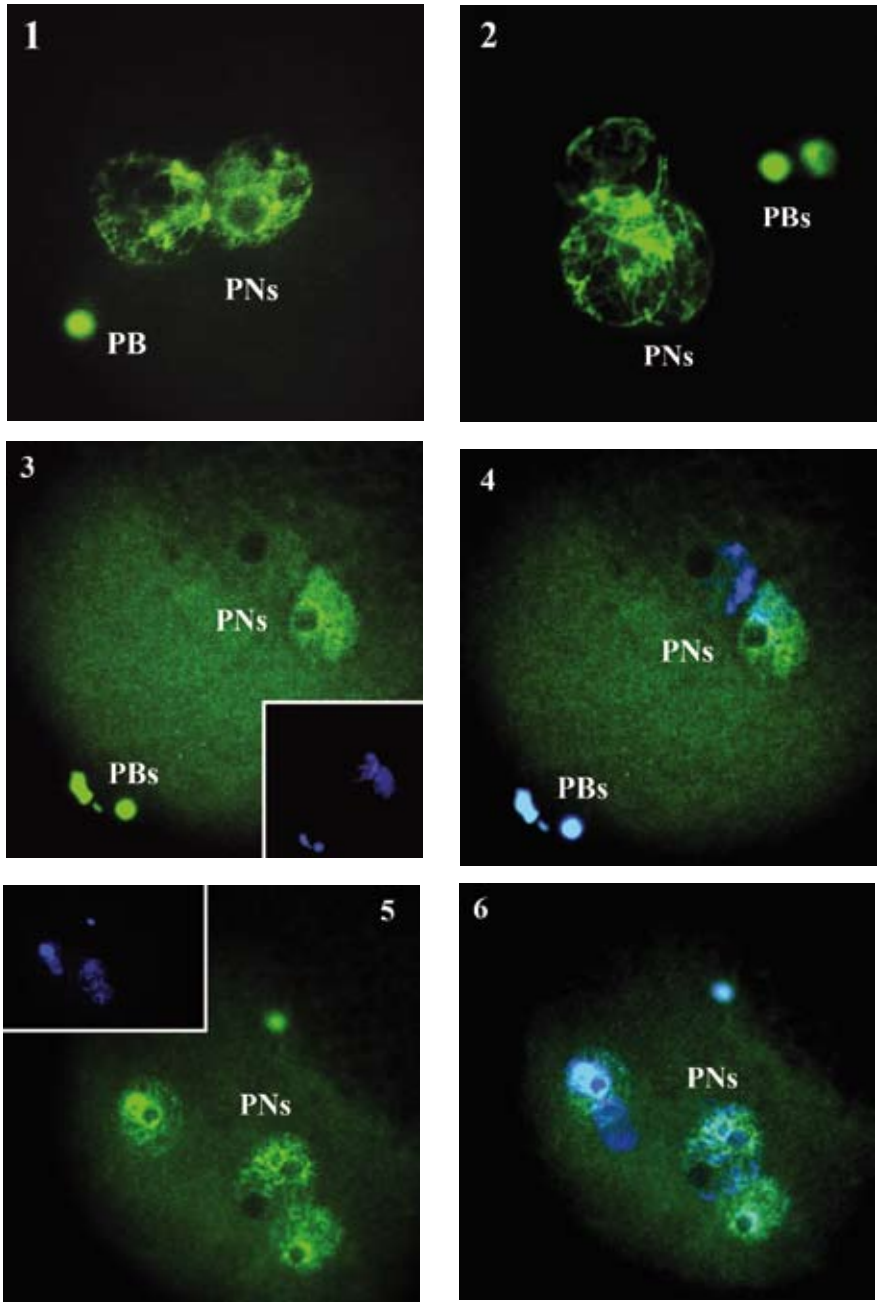

Figure 1. DNA methylation pattern in IVF porcine embryos
(1) 5-MeC (methylcytosine) labelling in monospermic embryos; both pronuclei are positively labelled
(2) 5-MeC labelling in polyspermic embryos; all pronuclei are positively labelled
(3) Dimethyl H3/K9 (histone 3/ lysine 9) labelling in monospermic embryo; one pronucleus is weakly labelled compared to the other one
(4) The same zygote as in (3); merged with DAPI
(5) dimethyl H3/K9 labelling in polyspermic embryos; all pronuclei are positively labelled
(6) The same zygote as in (5); merged with DAPI
PNs – pronuclei; PBs – polar bodies; blue – DAPI; green – 5-MeC or dimethyl H3/K9 antibody labelling

we used spermatozoa from several boars. When conventionally fertilized, the fertilization rate was highly variable, ranging from 10 to 80%, depending on the origin of boar spermatozoa and similarly variable was the monospermic or polyspermic penetration (20–60% of monospermic from fer-

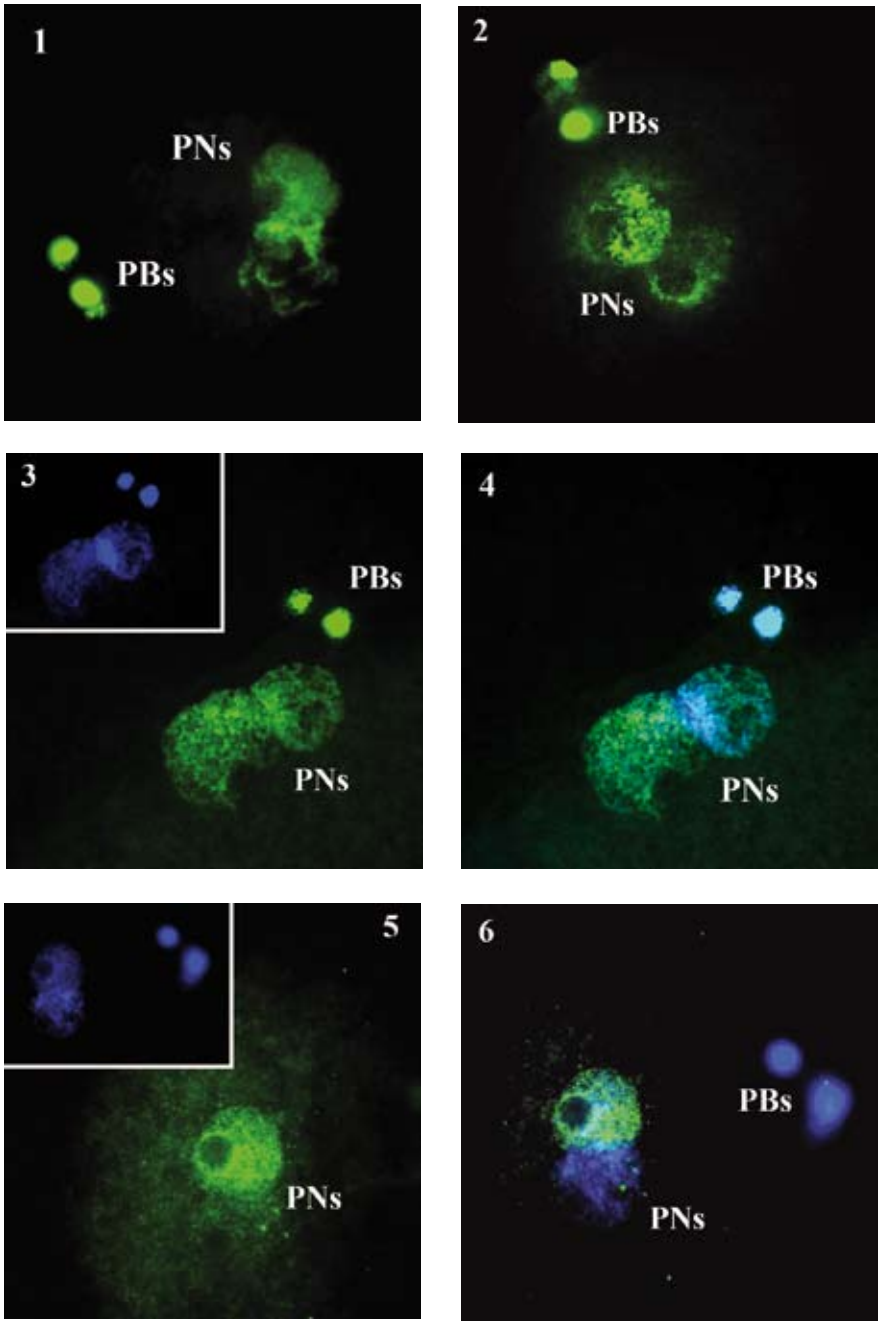

Figure 2. DNA methylation pattern in porcine embryos produced by intracytoplasmic sperm injection (ICSI)
(1) and 2) 5-MeC labelling in embryos produced by ICSI; both pronuclei are labelled
(3) Dimethyl H3/K9 labelling; both pronuclei are positively labelled
(4) The same zygote as in (3); merged with DAPI
(5) dimethyl H3/K9 labelling; only one pronucleus is labelled
(6) The same zygote as in (5); merged with DAPI
PNs – pronuclei; PBs – polar bodies; blue – DAPI; green – 5-MeC or dimethyl H3/K9 antibody labelling

tilized oocytes). Interestingly, in all *in vitro* fertilized oocytes, it does not matter if monospermic or polyspermic, the decondensation of sperm head(s) occurs and fully developed pronuclei were detected. This may indicate the acceptable quality of our oocyte culture conditions. The ICSI was typically successful in 94% (161/171), out of them 41% (66) of oocytes were with fully developed pronuclei, ma-

ternal and paternal. In the rest (95) the decondensation of sperm head was not complete or intact sperm heads could be detected along with the female pronucleus. All those zygotes also contained the second polar body. We assumed that the female pronucleus is in the vicinity to the second polar body. This, however, could be taken into consideration in only some labelled zygotes as polar bodies were often removed when zygotes were prepared for labelling (zona pellucida removal).

In total, we have successfully labelled 34 zygotes having two pronuclei after intracytoplasmic injection and 31 zygotes with two or more pronuclei after *in vitro* fertilization (Table 1). None of ICSI (monospermic) embryos (5-MeC labelling) exhibited complete demethylation in the paternal pronucleus. The embryos after *in vitro* fertilization (mostly polyspermic) exhibited the same 5-MeC labelling patterns, all pronuclei were positively labelled (Table 1, Figures 1 and 2).

The partial demethylation in the paternal pronucleus after H3/K9 labelling was observed in 42% (8) of embryos after *in vitro* fertilization and in 44% (11) of embryos after intracytoplasmic sperm injection. In these zygotes we have observed that one pronucleus is intensively labelled whilst the other one is almost without labelling. The remaining embryos showed the same level of labelling in both pronuclei (Table 1). Some embryos (35) were not labelled at all for unknown reasons. These embryos were eliminated from further evaluation (Table 1).

When comparing merged pictures (DAPI/specific antibody), it is clear that the given antibody typically labels chromatin. In some zygotes we detected more intensive labelling around the nucleolus(i) (Figures 1.1, 1.3, 1.5) or at the site of pronuclear apposition (syngamy, Figures 1.2, 2.1), in other zygotes the labelling was without any notable points (Figure 2.3).

We have not seen any significant differences between both these *in vitro* production techniques, i.e. conventional *in vitro* fertilization and intracytoplasmic sperm injection. The antibody against 5-MeC labelled both the paternal and the maternal pronuclear DNA both in IVF derived and in ICSI derived embryos. With H3/K9 partial paternal DNA demethylation (weaker labelling) was observed in about 40% of the evaluated embryos and this unequal methylation pattern was visible essentially at the same frequency in embryos produced by IVF and ICSI. From these results we can deduce that the embryo production techniques had no effect on the parental DNA methylation patterns (Table 1). Our data may, however, indicate some insufficiency of the oocyte maturation system used. But it is still unclear how important the paternal DNA demethylation is for further embryo development. The porcine zygotes produced by both these methods consistently develop up to the blastocyst stage (10–30%) in our lab.

DISCUSSION

In our study, the labelling of pronuclei in porcine zygotes with two different antibodies did not show any significant differences between zygotes produced by *in vitro* fertilization and by intracytoplasmic sperm injection. In the mouse there are also no differences from the aspect of methylation patterns between intracytoplasmic sperm injection, *in vitro* fertilization and *in vivo* produced embryos (Fulka and Fulka, 2006). Moreover, we did not detect the paternal DNA demethylation with 5-MeC antibody at all and only in about 40% of zygotes we were able to detect rather weaker labelling in male pronuclei with H3/K9 antibody. Similar results were published by Sega et al. (2007), who also demonstrated that male pronuclei in porcine zygotes frequently possess similar levels of H3/K9 labelling to those that can be found in female pronuclei.

The differences in the global demethylation pattern were first described by Mayer et al. (2000) and Oswald et al. (2000). Similar differences were demonstrated later on in some other species – bovine, rat, pig (Dean et al., 2001) and therefore it has been accepted that the global demethylation of paternal pronuclear DNA represents a common phenomenon typical of mammalian zygotes. This view has, however, been challenged later when sheep and rabbit pronuclear embryos (Beaujean et al., 2004; Shi et al., 2004) were labelled with the same antibody (5-MeC).

Similarly, contradictory results were obtained in porcine zygotes. Whereas some authors reported the demethylation of the paternal pronucleus (Dean et al., 2001; Fulka et al., 2006), others were unable to detect any differences in the intensity of labelling between both pronuclei (Jeong et al., 2007a,b). The reasons for these differences are rather unknown. Gioia et al. (2005) suggests that the important factor is in the origin of oocytes. If they mature completely *in vitro*, the frequency of paternal pronuclear DNA

demethylation is much lower when compared to those oocytes that underwent the germinal vesicle breakdown in follicles and thereafter complete the process of maturation in culture. This means that the quality of oocytes in the second group is much higher. However, the oocyte quality can be also affected by the technique of oocyte isolation (Wang et al., 2007).

Beside this, the ability of oocytes to form male pronuclei from sperm heads also differs between oocytes maturated *in vitro* and *in vivo*. The male pronucleus formation is delayed in most *in vitro* matured oocytes and zygotes thus present asynchronous pronuclei development when compared to *in vivo* matured oocytes (Laurincik et al., 1994). This asynchronous pronuclear formation can influence the epigenetic modifications of the genome. Goia et al. (2005) also observed the similar fluorescence intensity of methyl residues in male and female pronuclei in *in vitro* matured oocytes in spite of different results obtained with *in vivo* matured oocytes.

In general, the histone H3/K9 methylation is linked to the silencing of euchromatin and formation of heterochromatin (Lachner and Jenuwein, 2002). In addition to this, the H3/K9 position can be mono-, di- or tri-methylated and all these modifications can combine and act differently in gene activation or repression following the rules of a histone code (Strahl and Allis, 2000; Turner, 2000).

The presence of dimethyl group on H3/K9 could protect the female pronucleus from active demethylation while the absence of dimethylation on H3/K9 in the male pronucleus allows to the male pronuclear DNA to undergo the active demethylation (Santos et al., 2005). Sega et al. also supposed that DNA demethylation in *in vitro* produced porcine zygotes is not possible when H3/K9 is dimethylated. Jeong et al. (2007b) observed no demethylation of 5-MeC and dimethyl H3/K9 in the pig. What is more interesting, is that trimethylation of H3/K9 was established during pronucleus development and this process was independent of the DNA replication.

We do not know if different methylation pattern of *in vitro* matured oocytes is influenced by nonfunctional demethylases or by modified activities of methyltransferases. H3/K9 became methylated in pig zygote due to the activity of different histone methyltransferases (HMTases). These activities may be different in the mouse, where the HMTases are inhibited or inactive, because the newly incorporated histones into the male pronucleus do not become methylated on H3/K9 (Jeong et al., 2007b). In the mouse the demethylation begins almost immediately after fertilization, when protamines are replaced with oocyte histones, whereas in some other species (pig, rabbit) the demethylation processes occur more slowly and only when pronuclei are fully developed.

Also in human zygotes there was only partial or no demethylation observed after fertilization in zygotes originating from a patient who underwent assisted fertilization (Fulka et al., 2004), whilst other authors reported the complete demethylation of paternal genome (Beaujean et al., 2004). We do not know if the difference in methylation levels between zygotes is influenced by the oocyte and sperm quality of patients or by the own *in vitro* technique.

The processes of genome methylation and demethylation are rather complex and they are not completely known.

We have to take into consideration that pig oocytes matured *in vitro* may posses certain incompetence when compared to *in vivo* matured oocytes. The main problem is probably in insufficient cytoplasmic maturation. Thus, some proteins that are essential for paternal demethylation are not sufficiently functional or their activity is modified. We cannot however exclude some minor technical aspects that may possibly influence the overall results of labelling.

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