Dynamics of epigenetic remodeling in interspecies porcine zygotes

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ABSTRACT: The process of active paternal chromatin demethylation after fertilization in the pig is not fully understood and very inconsistent data have been published by different research groups. We have applied the interspecies intracytoplasmic sperm injection (iICSI) to evaluate remodeling capabilities of porcine oocytes in more details. We injected mouse frozen-thawed sperm heads into porcine *in vitro* matured or ovulated oocytes, respectively. Embryos produced by intracytoplasmic sperm injection (ICSI) of boar spermatozoa into porcine ovulated oocytes (intraspecies) served as controls. Zygotes with 2-pronuclei were labeled with antibodies against certain epigenetic modifications (5-methylcytosine, 5-MeC; heterochromatin protein 1, HP1; trimethylation of H3/K4, H3/K4-me3; and dimethylation of H3/K9, H3/K9-me2). The labeling patterns were not different between zygotes produced from *in vitro* matured and ovulated oocytes. Both pronuclei were symmetrically labeled with 5-MeC, HP1, and H3/K4-me3 antibodies. Asymmetrical labeling was observed only with H3/K9-me2 antibody. The labeling of interspecies zygotes was similar to that of intraspecies zygotes. Moreover, the DNA demethylation was observed neither in control zygotes (intraspecies). The only difference observed between zygotes produced from *in vitro* matured and ovulated oocytes was in their ability to be activated. Intraspecies zygotes produced from ovulated oocytes were able to form the paternal pronucleus without additional activation; the zygotes produced from *in vitro* matured oocytes formed the paternal pronucleus only after additional activation with electric pulses. Our results show that the remodeling abilities of *in vitro* matured and ovulated oocytes are essentially similar. Moreover, it seems that reasons of inconsistent data reporting the active demethylation in the pig are more complicated and they are not associated exclusively with the oocyte quality.

Keywords: pig; methylation; iICSI; zygote

After fertilization the maternal and paternal genomes undergo distinct types of modifications. The maternal genome completes meiotic division whilst the paternal genome undergoes radical morphological changes (Wright and Longo, 1988; Adenot et al., 1997). These morphological changes are accompanied by the replacement of protamines by histones. Simultaneously, a new nuclear envelope is formed around both decondensing chromatins and some additional proteins are imported from the ooplasm into pronuclei (Schatten et al., 1988). Although both pronuclei are formed in the same cytoplasm, several epigenetic differences are evident between them. The first difference is a hyperacetylation of histone H4 in the paternal pronucleus. This difference disappears before DNA replication begins (Adenot et al., 1997). The second difference is the demethylation of the paternal pronucleus genome which appears shortly after fertilization. It is commonly accepted that the paternal genome is actively demethylated in most of mammals whereas the maternal genome

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remains methylated (Dean et al., 2001; Lepikhov et al., 2008).

The role of active DNA demethylation is not fully understood. As mentioned above, it is commonly accepted that active demethylation of the paternal genome is a common phenomenon in all mammals (Dean et al., 2001). The paternal pronucleus of mouse zygote is extensively demethylated, while the maternal genome remains highly methylated (Oswald et al., 2000; Santos et al., 2005). Similar results were reported also for rat and monkey zygotes (Yang et al., 2007; Zaitseva et al., 2007). However, later on, this assumption was changed by some additional experiments. It has been shown in the rabbit and also in the sheep that both pronuclei posses the same level of methylation (Beaujean et al., 2004a). Besides this, much less extensive demethylation has been detected in bovine, goat, and human zygotes (Fulka et al., 2004; Park et al., 2007; Abdalla et al., 2009). The role of active demethylation of paternal pronucleus has been challenged again by Lepikhov et al. (2008). Their last published results show that even in the rabbit there is an evident demethylation of paternal genome at advanced stages of zygotes (Lepikhov et al., 2008).

Different results have been published for porcine zygotes. Here, in naturally fertilized embryos, an extensive demethylation of paternal DNA has been reported (Dean et al., 2001; Fulka et al., 2006). In contrast, the paternal genome was not demethylated in zygotes from *in vitro* matured oocytes (Jeong et al., 2007b; Barnetova and Okada, 2010). As shown additionally, the oocyte demethylation activity depends on the quality of oocyte maturation, namely on the period of germinal vesicle breakdown (Gioia et al., 2005). In these oocytes matured *in vivo* or where the oocytes were isolated from follicles after germinal vesicle breakdown (GVBD), the paternal genome was extensively demethylated. On the other hand, in the majority of oocytes that were completely matured *in vitro*, no such prominent changes have been detected (Gioia et al., 2005). The oocyte quality is one of the aspects which can explain the reasons of inconsistent results observed in porcine zygotes. However, it seems that the active demethylation of paternal genome is absent also in high quality oocytes. As shown in the latest published paper, the active demethylation was not observed either in *in vivo* produced zygotes (Deshmukh et al., 2011).

In our experiment we have used the interspecies sperm injection (iICSI). This method allows us to divide experimentally the sperm and oocyte contribution to the paternal genome remodeling after fertilization. In our previous work we have injected porcine sperms into mouse ovulated or *in vitro* matured oocytes (Barnetova et al., 2010). The paternal genome of porcine origin was demethylated in mouse ovulated oocytes. On the other hand, the paternal genome decondensation did not occur in mouse *in vitro* matured oocytes. Therefore we were not able to use these zygotes for parallel evaluation. Here we have used a reversed approach – mouse sperm heads injection into porcine oocytes.

MATERIAL AND METHODS

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

Ovulated and *in vitro* matured porcine oocytes

Prepubertal, 6 months old gilts, White Landrace × SL 48 hybrid (White Landrace × Hampshire) were stimulated by 1000 IU pregnant mare serum gonadotropin (PMSG, Serva, Bioveta, a.s., Ivanovice na Hané, Czech Republic) and 72 h later by 2 ml (50 μg/ml) gonadotropin releasing hormone (GnRH, Depherelin Gonavet Veyx, Frenštát pod Radhoštěm, Czech Republic). Ovulated oocytes were recovered by flushing oviducts with phosphate buffered saline about 5 h after the expected time of ovulation. Oocytes were denuded from cumulus cells by incubating them briefly in 0.01% hyaluronidase followed by pipetting through a small-bore pipette and immediately used for porcine/mouse sperm injection. A minority of oocytes was also isolated from ovaries of stimulated gilts that did not ovulate. The oocytes were aspirated from large follicles and cultivated *in vitro* for next 22 h to reach metaphase II. The oocytes are noted in the text as partly *in vitro* matured oocytes.

Porcine immatured oocytes were collected from medium-size antral follicles. Groups of 25 to 35 oocyte-cumulus-complexes were cultured in 500 μl of bicarbonate-buffered medium 199 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 44–46 h in a humidified atmosphere of 5% CO_2 in air at 38.5°C. After maturation the oocytes were denuded and used for sperm injection.

Porcine/mouse spermatozoa injection and activation of oocytes

Porcine oocytes were injected on the inverted microscope stage (Olympus IX-70) with PMM piezo injection system essentially as described by Kimura and Yanagimachi (1995), in drops of HTF-HEPES medium (Cambrex Bio Science, Verviers, Belgium). Porcine spermatozoa suspension, preincubated in mTBM medium for 30 min in incubator to induce capacitation and acrosomal reaction, was added to a 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). Motile spermatozoa were immobilized by hitting the mid-piece by the injection pipette, aspirated into the pipette, and injected into the oocyte. Heads from mouse frozen-thawed spermatozoa were separated from the tail by the injection pipette and the isolated sperm heads were injected into oocytes.

As the injected *in vitro* matured oocytes were very only exceptionally activated, it was necessary to activate them additionally with one electric pulse in 0.3M mannitol, 0.05mM $CaCl_2$, and 0.01mM $MgSO_4$ (100 µs, 1.5 kV/cm).

The injected oocytes were cultured for 15–22 h in 20 µl drops of the cultivation medium PZM3 supplemented with 3 mg/ml BSA, covered with paraffin oil (Carl Roth, Karlsruhe, Germany). Each group contained ten oocytes.

Fixation and antibody labeling

The zygotes were incubated for 10 min in HTF-HEPES medium (Cambrex Bio Science, Verviers, Belgium) supplemented with cytochalasin B (7.5 µg per ml) and then centrifuged for 10 min at 9000 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 pre-fixed in 4% paraformaldehyde for 1 h and fixed in 4% paraformaldehyde with 0.01% Triton X-100 overnight. Fixed oocytes were used for antibody labeling with following antibodies: Pan Histone (Roche, Prague, Czech Republic); anti-dimethylation on lysine 9 of histone 3 (H3/K9-me2, Upstate, Charlottesville, USA), anti-heterochromatin protein 1 (HP1, Abcam, Cambridge, UK), anti-trimethylation on lysine 4 of histone 3 (H3/K4-me3, Abcam, Cambridge, UK), and anti-5-methylcytosine (5-MeC, Eurogentec, Seraign, Belgium). The labeling and evaluation steps were essentially the same as described by Fulka et al. (2008). Briefly, the zygotes were permeabilized in 0.2% Triton X-100 in phoshate-buffered saline (PBS) for 20 min. For anti-5-MeC labeling, the zygotes were washed in PBS/1% BSA, then transferred to 2M HCl for 30 min and washed again in PBS/1% BSA. Consequently the samples were blocked for 2 h in 0.2% Triton X-100/5% Normal Goat Serum (Santa Cruz Biotechnology, Santa Cruz, USA) in PBS. After blocking the samples were incubated with the primary antibody in the blocking buffer at 4°C overnight. For the other antibodies, after fixation and permeabilization the samples were blocked in 5% Normal Goat Serum (Santa Cruz Biotechnology, Santa Cruz, USA) in PBS for 2 h. Consequently the samples were incubated with the appropriate first antibody diluted in blocking solution at 4°C overnight.

After incubation with the primary antibody the samples were washed in PBS/1% BSA and incubated with the secondary antibody conjugated with FITC for 2 h at room temperature. After the incubation with the secondary antibody the samples were washed again (PBS/1% BSA), mounted in SlowFade Gold (Invitrogen, Carlsbad, USA) mounting medium, and examined.

Fluorescence *in situ* hybridization (FISH)

Fluorescence *in situ* hybridization (FISH) was used to identify the paternal nucleus after the mouse sperm injection into porcine *in vitro* matured oocytes. Mouse Cot1 DNA was used to detect specifically mouse DNA. Mouse Cot1 DNA (Invitrogen, Carlsbad, USA) was labeled by Fluorescein-High Prime random priming labeling mix (Roche, Prague, Czech Republic) and then used as a probe. The interspecies zygotes were washed in a drop of 0.01M HCl/0.1% Tween-20 spreading solution and then placed on a slide in a drop of the same medium. After spreading, the zygotes were

fixed by methanol: acetic acid (3:1), air-dried, and stored in 70% ethanol at –20°C.

The slides were rehydrated in 2 × SSC before RNase treatment. RNase treatment was applied for 30 min at 37°C. Then the slides were dehydrated and treated by pepsin. After that the slides were dehydrated again through ethanol series, co-denatured with the labeled Cot1 probe in 50% formamide, 10% dextran sulphate in 2 × SSC, and left to hybridize overnight in a humidified chamber at 37°C. After hybridization the slides were washed 3 × 10 min in 2 × SSC/50% formamide, 0.1 × SSC and 4 × SSC/0.1% Tween-20 at 42°C. Than the slides were mounted in SlowFade Gold antifade reagent with DAPI (Invitrogen, Carlsbad, USA) and evaluated.

Quantitative analysis

The zygotes were evaluated under the Olympus BX61 fluorescence microscope and pictures were adjusted by AnalySIS (Soft Imaging System GmbH, Munster, Germany) and Adobe Photoshop Software. The fluorescence intensities of maternal and paternal pronuclei were measured using ImageJ Software. After subtraction of cytoplasm background, each pronucleus was manually outlined and exposed to measure the fluorescence intensity. The mean of fluorescence intensity was calculated as the ration of total fluorescence intensity in the outlined pronucleus to the area. Consequently, the relative fluorescence intensities were calculated as the ratio of means of fluorescence intensities of pronucleus with the higher mean of fluorescence intensity to the pronucleus with the lower mean of fluorescence intensity. Student's *t*-test was used to compare the data obtained from the pictures taken after each antibody labeling.

RESULTS

Frozen-thawed mouse sperm heads were either injected into porcine *in vitro* matured or ovulated oocytes. As controls we have injected porcine spermatozoa into ovulated oocytes. Porcine *in vitro* matured oocytes were used for boar spermatozoa injection in our previous experiments (Barnetova and Okada, 2010). Data obtained in this study were thus compared with data from the previous work.

We have injected 631 *in vitro* matured oocytes with mouse sperm heads. As mouse sperm heads did not decondense at all in the oocyte cytoplasm without additional activation of oocytes (data not shown), it was necessary to activate the injected zygotes with the electric pulse. After the activation, we have detected both pronuclei (the paternal and maternal pronucleus, Table 1) in 30.6% of

Table 1. Activation rates in zygotes produced from *in vitro* matured, partly *in vitro* matured and ovulated oocytes. The activation ability of ovulated oocytes is higher than the ability of *in vitro* matured oocytes. The difference between *in vitro* matured and ovulated oocytes is evident mainly in interspecies zygotes. The interspecies zygotes produced from ovulated oocytes are able to form the paternal pronucleus without any additional activation. This is in contrast to interspecies zygotes produced from *in vitro* matured oocytes, where no pronuclei were formed (0% of activation) without the activation with the electrical pulse. The difference in pronuclei formation between oocytes in intraspecies zygotes is not significant; however, in ovulated oocytes there is a tendency to have the higher activation ability (in %)

Oocytes according to the maturation	ICSI (porcine oocyte and boar sperm)			iICSI (porcine oocytes and mouse sperm head)		
	no. of injected oocytes	no. of 2-pronuclei zygotes	activation rate† (in %)	no. of injected oocytes	no. of 2-pronuclei zygotes	activation rate† (in %)
IVM[a]	161	66	41	631*	193	30.6
Partly IVM	23	9	39.1	24*	7	29.2
Ovulated	39	20	51.2	85	32	37.6

IVM = *in vitro* matured oocytes; partly IVM = maturation *in vitro* only for 22 h
*additional activation was necessary for pronuclei formation (electrical pulse)
†the statistical comparison of pronuclei formation with ovulated oocytes did not confirm a significant difference (χ^2-test = 1.356, 0.858, 1.733, 0.586 for IVM-ICSI, partly IVM-ICSI, IVM-iICSI, and partly IVM-iICSI, respectively; $P > 0.05$)
[a]Barnetová and Okada (2010)

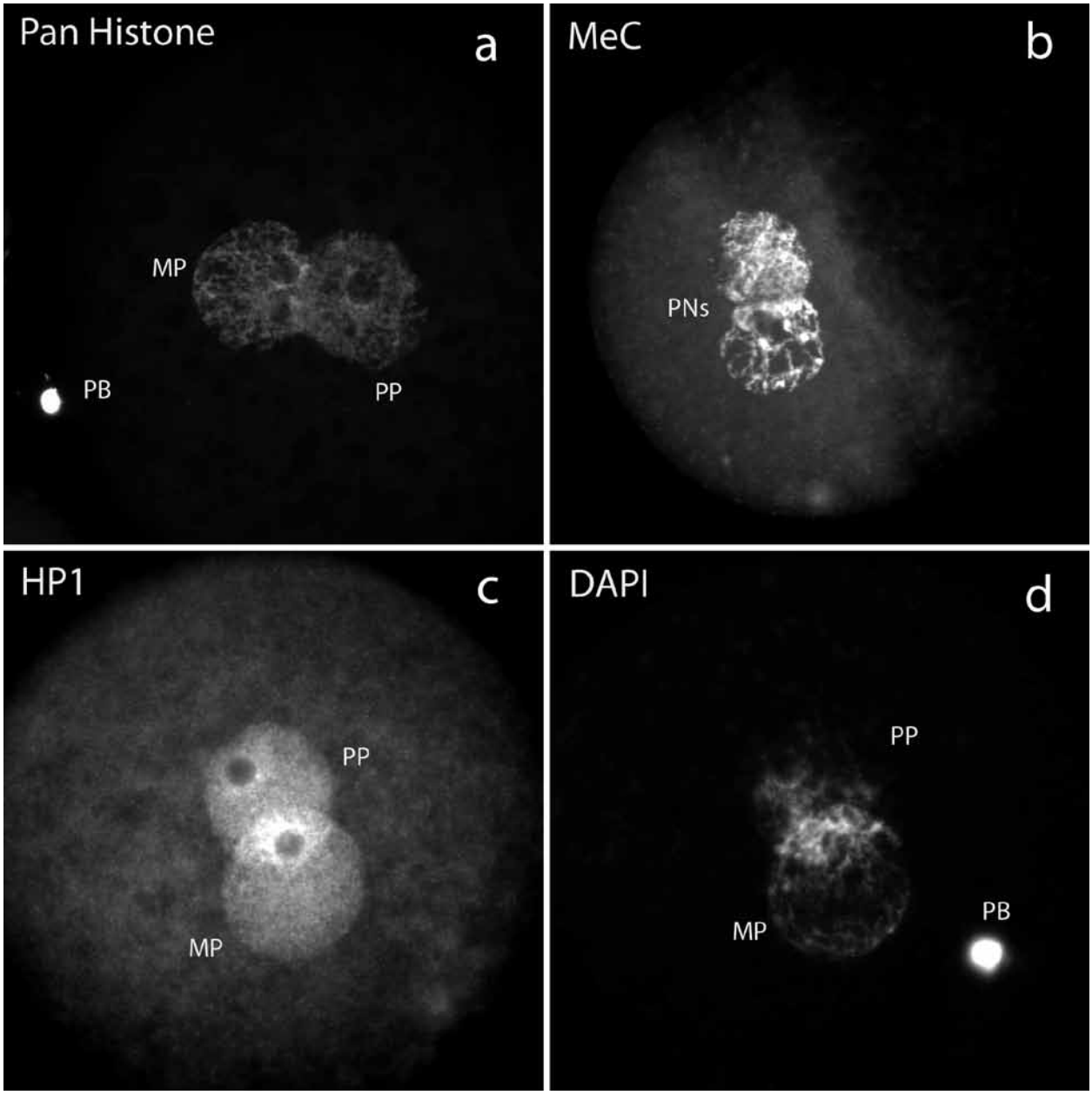

Figure 1. Pan Histone, 5-MeC and HP1 labeling in interspecies zygotes (mouse sperm heads injected into porcine oocytes). All the zygotes used for evaluation were in the stage of pronuclei apposition

(a) labeling of interspecies zygotes against Pan Histone showed positive labeling in both pronuclei (sperm protamines were replaced by oocyte histones), (b) labeling with 5-MeC antibody showed positive labeling of both pronuclei (no demethylation of paternal pronucleus); (c–d) labeling with HP1 antibody showed symmetrical labeling: (c) both pronuclei were positively labeled, (d) parallel DAPI staining

MP = maternal pronucleus, PP = paternal pronucleus, PB = polar body, PNs = pronuclei

presumptive zygotes. Both pronuclei had the same size which is typical also of porcine intraspecies zygotes. In the rest, mouse sperm heads did not decondense, the oocytes degenerated, or only one pronucleus (maternal) was detected.

To confirm the origin of the pronuclei, namely those originated from the injected mouse sperm heads (and to exclude the possibility that pronuclei might originate from, for example, fragmented maternal pig genome), we have performed a species-specific fluorescence *in situ* hybridization (FISH). The fluorescently labeled probe was generated from mouse Cot1 DNA. We have used oocytes containing non-decondensed mouse sperm heads as controls.

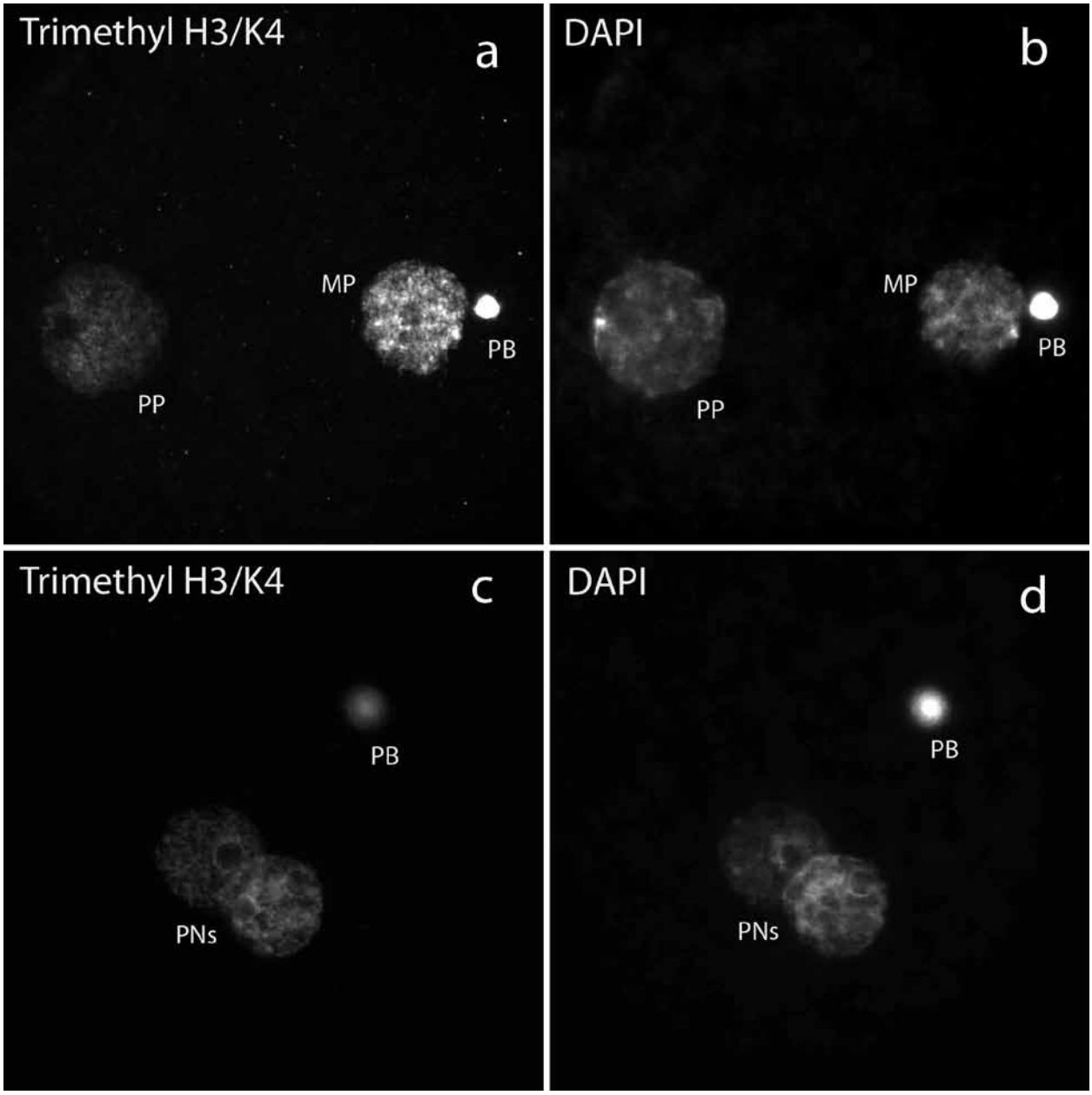

Figure 2. H3/K4-me3 labeling in interspecies zygotes (mouse sperm heads injected into porcine oocytes). The zygotes used for evaluation were in the stage of early pronuclei formation (a–b) and in the stage of pronuclei apposition (c–d)
(a–b) labeling of interspecies zygotes with H3/K4-me3 in 7 h after sperm head injection: (a) the paternal pronucleus is weakly labeled compared to the maternal pronucleus, (b) parallel DAPI staining; (c–d) labeling of interspecies zygotes with H3/K4-me3 in 22 h after injection: (c) both pronuclei are positively labeled, (d) parallel DAPI staining
MP = maternal pronucleus, PP = paternal pronucleus, PB = polar body, PNs = pronuclei

In this case, the species-specific hybridization was selectively detected in non-decondensed mouse sperm heads and no hybridization was detected in the maternal (porcine) pronucleus. In the case where two pronuclei were present in interspecies zygotes, only one of them hybridized readily with the mouse-specific probe (Figure 3c, d). Thus, our results clearly confirmed the presence of paternal mouse DNA.

In the other part of the study, we have injected 85 ovulated oocytes with mouse sperm heads. Both pronuclei were detected in 37.6% of zygotes (Table 1). Contrary to the previous part of experiments, the oocytes were activated without any ad-

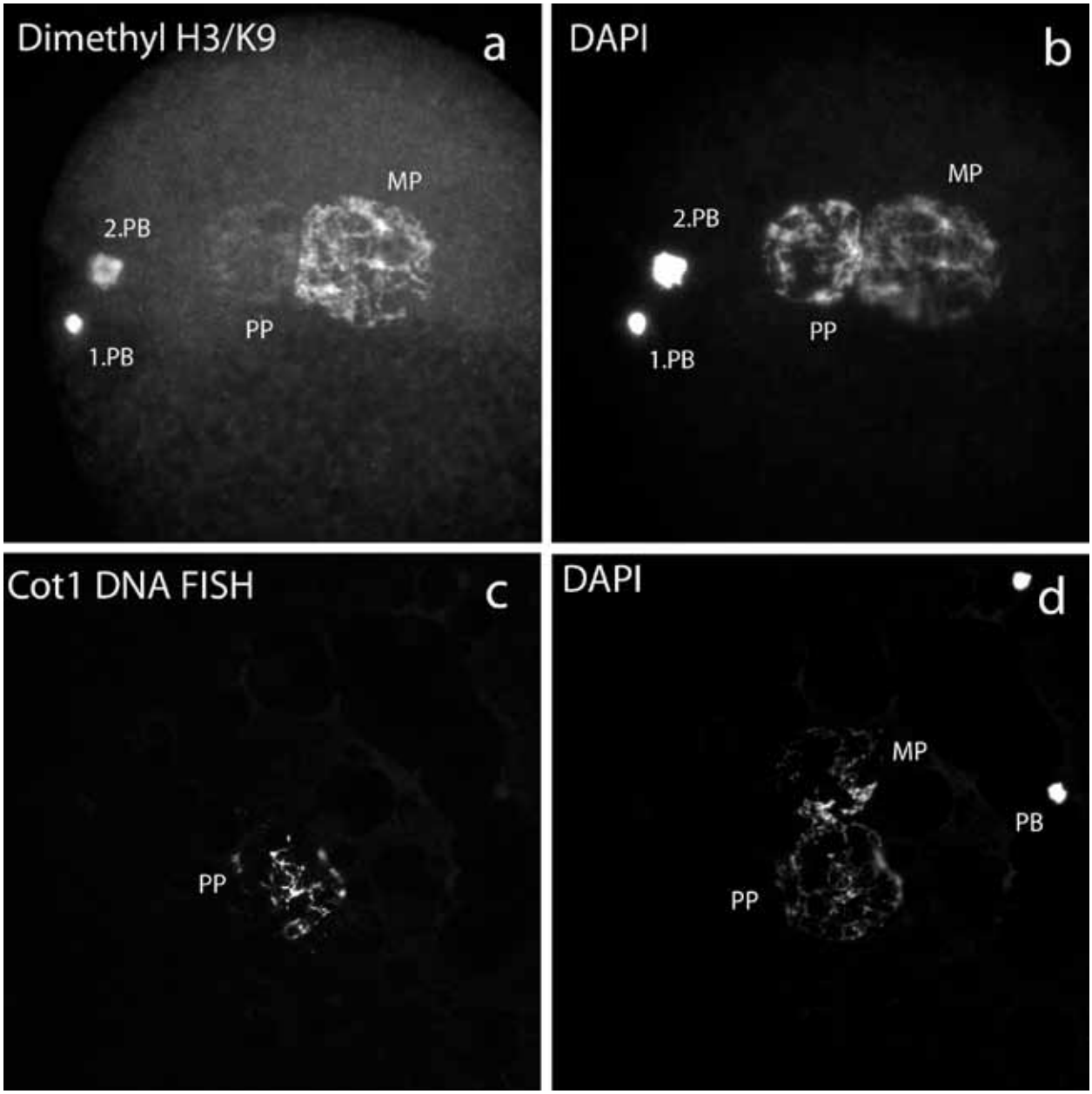

Figure 3. H3/K9-me2 labeling in interspecies zygotes and the verification of the male pronucleus origin (mouse sperm heads injected into porcine oocytes). All the zygotes used for evaluation were in the stage of pronuclei apposition
(a–b) asymmetrical labeling of interspecies zygotes with H3/K9-me2: (a) the paternal pronucleus is not methylated, (b) parallel DAPI staining; (c–d) interspecies zygotes were hybridized with mouse FITC labeled Cot1 DNA: (c) positive signal was detected only over the paternal pronucleus, (d) parallel staining with DAPI confirms the presence of both pronuclei
MP = maternal pronucleus, PP = paternal pronucleus, PB = polar body

ditional stimulus. A minority of oocytes, which did not ovulate in stimulated gilts although they started to mature, were cultivated for another 22 h *in vitro* to reach MII. These oocytes were also injected with mouse sperm heads; however, an additional activation was necessary for successful pronuclei formation (Table 1).

To evaluate the possible affect of parthenogenetic activation on the final epigenetic remodeling, we applied electrical pulses on injected ovulated oocytes. There was no difference between the injected oocyte and the injected activated one either in the rate of pronuclei formation or in the epigenetic remodeling (data not shown).

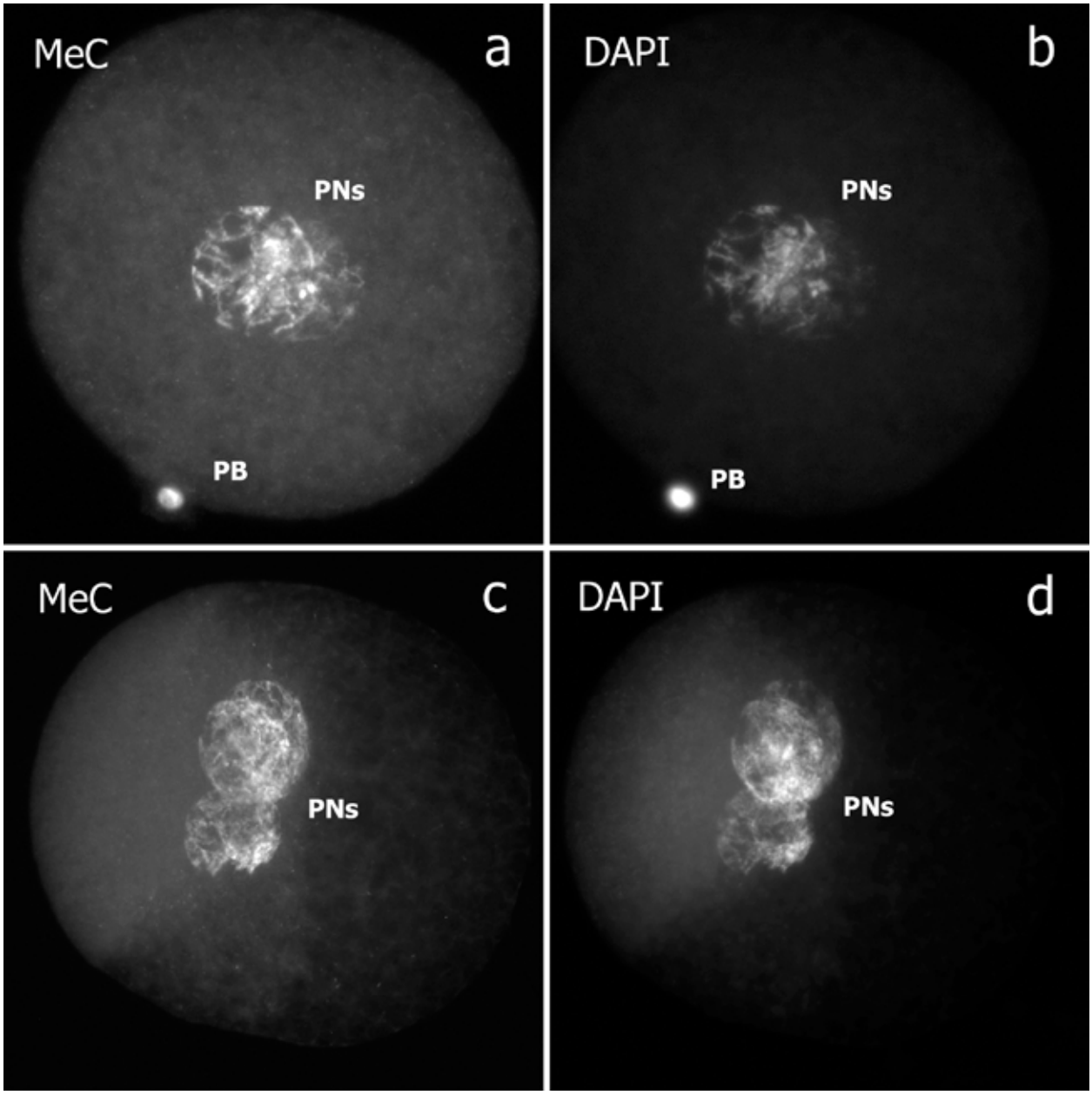

Figure 4. DNA methylation patterns in intraspecies zygotes produced from *in vitro* matured and ovulated oocytes. There is no DNA demethylation in any type of zygotes. All the zygotes used for evaluation were in the stage of pronuclei apposition (a–b) 5-MeC labeling in 22 h after the injection of porcine spermatozoa into the ovulated oocyte: (a) 5-MeC labeling, (b) parallel DAPI staining; (c-d) 5-MeC labeling in 22 h after the injection of porcine spermatozoa into *in vitro* matured oocyte: (c) 5-MeC labeling, (d) parallel DAPI staining

The reconstructed zygotes were used in the stage of pronuclei apposition for antibody labeling of Pan Histone and some epigenetic marks (5-MeC, HP1, H3/K9-me2, H3/K4-me3). The labeling with anti-Pan Histone antibody showed that both pronuclei were positively labeled (Figure 1a). This labeling clearly confirmed the removal of mouse sperm protamines and their replacement by porcine oocyte specific histones. Thus, these results show that the porcine oocyte is able to induce the paternal pronucleus formation of the sperm head of another species (mouse). The labeling with anti-5-MeC did not show any demethylation of paternal pronucleus (Figure 1b). Both pronuclei were labeled in the same level (at 22 hpf) in both types of zygotes (from *in vitro* matured and ovulated oocytes). The

Table 2. Statistical analysis of fluorescence intensities of maternal and paternal pronuclei in interspecies zygotes. Only zygotes with considerably formed pronuclei and clear antibody labeling were used for the analysis. There was no significant difference between 5-MeC, HP1 and H3/K4-me3 labeling in both types of interspecies zygotes (from ovulated and *in vitro* matured oocytes; both pronuclei labeled with the same intensity). The only significant difference in fluorescence intensity between pronuclei was after the labeling with H3/K9-me2 (maternal pronucleus labeled, paternal pronucleus unlabeled)

Antibody	*In vitro* matured oocytes		Ovulated oocytes	
	no. of analyzed pictures	Relative fluorescence intensity (mean ± SEM)	no. of analyzed pictures	Relative fluorescence intensity (mean ± SEM)
5-MeC	10	1.107 ± 0.02[a]	5	1.184 ± 0.04[a]
HP1	9	1.154 ± 0.05[a]	6	1.097 ± 0.03[a]
H3/K9-me2	10	1.513 ± 0.1[b]	3	1.974 ± 0.28[b]
H3/K4-me3	7	1.167 ± 0.05[a]	3	1.108 ± 0.02[a]

5-MeC = 5-methylcytosine; HP1 = heterochromatin protein 1; H3/K9-me2 = dimethylation on lysine 9 of histone 3; H3/K4-me3 = trimethylation of lysine 4 on histone 3
[a,b]significant difference at $P < 0.05$

demethylation was not observed even in control zygotes (intraspecies) produced by injection of porcine spermatozoa into ovulated oocytes (Figure 4). These intraspecies zygotes were fixed in different times after injection (16, 20, 22 h) and in all of them both pronuclei were labeled in the same level (data not shown). At 22 hpf some intraspecies zygotes (about 30%) started to divide and the rest of zygotes showed the progressed pronuclear apposition (Figure 4). The labeling with anti-5-MeC antibody did not show the active demethylation in porcine zygotes even in the progressed stage of development. Labeling patterns with remaining antibodies were also the same in both types of zygotes (from ovulated and *in vitro* matured oocytes). Both pronuclei were labeled in the same intensity with anti-HP1 antibody (Figure 1c, d). The paternal pronucleus was weakly labeled with anti-H3/K9-m2 antibody (Figure 3a, b). With anti-H3/K4-me3 labeling the paternal pronucleus was weakly labeled in 7 h after injection (early stage of pronuclei); however, in 22 h after injection both pronuclei were

Table 3. Chromatin characteristics of paternal pronuclei in intra- and interspecies ICSI zygotes produced by porcine/mouse sperm heads injection into porcine oocytes. There was no difference in the epigenetic labeling between interspecies zygotes produced from *in vitro* matured and ovulated oocytes. The labeling of interspecies zygotes was similar to patterns typical of intraspecies zygotes

Antibody	Porcine intraspecies zygotes (porcine oocyte and boar sperm)		Porcine interspecies zygotes (porcine oocyte and mouse sperm)	
	MP	PP	MP	PP
Pan Histone	+	+	+	+
5-MeC	+	+	+	+
HP1	+	+	+	+
H3/K9-me2	+	–[a]	+	–
H3/K4-me3	+	+[b]	+	+*

MP = maternal pronucleus; PP = paternal pronucleus; 5-MeC = 5-methylcytosine; HP1 = heterochromatin protein 1; H3/K9-me2 = dimethylation on lysine 9 of histone 3; H3/K4-me3 = trimethylation of lysine 4 on histone 3
+ = positive labeling; – = weak/asymmetrical labeling
*labeling in 7 h after injection (intermediate labeling) is different than in 22 h after injection (positive labeling)
[a]variable antibody labeling, the pronucleus is sometimes labeled and sometimes not labeled (Barnetova and Okada, 2010; Sega et al., 2007)
[b]Jeong et al. (2007b)

positively labeled (pronuclei apposition, Figure 2). Quantitative analysis of fluorescence intensities in interspecies zygotes confirmed our observation (Table 2). The same labeling was typical also of control intraspecies zygotes (Table 3).

Our results prove that *in vitro* matured and ovulated oocytes differ in their activation ability; however, their epigenetic remodeling potential is essentially the same. Moreover, porcine oocytes cytoplasm seems to be able to epigenetically remodel a chromatin of other species. The paternal pronucleus of other species (mouse) is epigenetically remodeled in the way which is typical of intraspecies porcine zygotes.

DISCUSSION

The iICSI is a powerful technique that allows us to experimentally divide the paternal and maternal pattern of genome remodeling in zygotes. The iICSI was first used by Beaujean et al. (2004b) for studies of epigenetic remodeling abilities of oocytes. Beaujean et al. injected, for example, mouse sperm heads into ovine oocytes. The paternal pronucleus of mouse origin was partly demethylated in spite of absence of active demethylation in ovine intraspecies zygotes (Beaujean et al., 2004b). On the other hand, the ovine sperm was only partly demethylated in mouse oocytes although the extensive demethylation is typical of the mouse (Beaujean et al., 2004b). This intermediate methylation pattern was interpreted as that the final methylation status is the result of both parts – the cytoplasm of oocyte and sperm factors. According to our results it seems that it is mainly affected by the oocyte cytoplasm. The paternal pronucleus of mouse origin was not demethylated at all in the porcine oocyte. The absence of demethylation was seen also in porcine intraspecies zygotes. The labeling with other antibodies (HP1, H3/K9-me2, H3/K4-me3) in interspecies porcine zygotes was also similar to the pattern typical of intraspecies zygotes (symmetrical labeling of HP1 and H3/K4-me3, asymmetrical labeling of H3/K9-me2). In addition, in porcine interspecies zygotes both pronuclei were of the same size as is typical of intraspecies porcine zygotes. Moreover, in our previous study we injected porcine spermatozoa into mouse oocyte and these spermatozoa underwent active demethylation (Barnetova et al., 2010). The labeling with the same antibodies in mouse interspecies zygotes was also more similar to the mouse intraspecies zygotes (asymmetrical labeling for HP1, H3/K4-me3 and H3/K9-me2). Besides, in mouse zygotes the paternal and the maternal pronuclei differ in their size. The paternal pronucleus is larger when compared with the maternal pronucleus. This phenomenon was also observed in mouse interspecies zygotes (Barnetova et al., 2010). Therefore it seems that the epigenetic remodeling and the regulation of pronuclei size is the property of oocyte cytoplasm.

The difference between *in vitro* matured and ovulated oocytes, which we observed, was the activation ability of oocytes. Both the completely and the partly *in vitro* matured oocytes were not capable of forming paternal pronucleus of mouse origin (0% of activation). These oocytes formed the pronucleus only after additional activation with the electric pulse. In contrast, ovulated oocytes formed the pronucleus of mouse origin without any additional activation. Nevertheless, the electrical activation of oocytes enabled us to eliminate the difference in activation rates in interspecies zygotes (no significant difference in activation rates in iICSI; Table 1). In contrast, in intraspecies zygotes the difference in activation ability between *in vitro* matured and ovulated oocytes is not so evident. According to our previous results observed in intraspecies zygotes produced from *in vitro* matured oocytes (Barnetova and Okada, 2010) and the last results observed in control zygotes produced from ovulated oocytes, the difference in activation ability of oocytes in intraspecies zygotes was not confirmed significantly as well (Table 1). It must be noted that all the ICSI zygotes formed pronuclei without any additional activation. Comparable situation we observed in the study with mouse interspecies zygotes (Barnetova et al., 2010). There, mouse ovulated oocytes formed the paternal pronucleus of porcine origin in the high rate. Nevertheless, when *in vitro* matured oocytes were used, the paternal pronucleus was not formed at all. The paternal pronucleus of porcine origin was formed neither after additional activation of the oocytes by $SrCl_2$, nor after the treatment of spermatozoa (Triton X-100, freezing, or sonication of sperm; Barnetova, unpublished).

The role of active demethylation is not fully determined. Beyond, in the pig, quite inconsistent data have been published. Dean et al. (2001) and Fulka et al. (2006) observed the active demethylation in porcine zygotes. In contrast, other authors did not observe the active demethylation at

all (Jeong et al., 2007a). It has been thought that the demethylation ability of the oocyte is dependent on the oocyte quality. The oocyte completely matured *in vitro* is not capable to demethylate the paternal genome. Nevertheless, when oocytes are partly or completely matured *in vivo*, the paternal pronucleus is demethylated (Gioia et al., 2005). In our study we have used ovulated oocytes to check this statement. In spite of the obvious difference in the quality of *in vitro* matured and ovulated oocytes, the epigenetic remodeling potential seems to be the same. We did not observe the demethylation of paternal pronucleus in interspecies zygotes from both types of oocytes (*in vitro* matured and ovulated). What is more, there was no active demethylation in control zygotes produced from ovulated oocytes. Our observations of DNA methylation are in agreement with the work of Deshmukh et al. (2011). Authors evaluated the methylation pattern of naturally produced zygotes with no demethylation of paternal genome observed.

The reason of inconsistent data observed in porcine zygotes still remains unclear. In our conditions the final remodeling was not affected by the technique used for embryo production (Fulka et al., 2006; Barnetova and Okada, 2010). Moreover, sperm head was differentially remodeled in cytoplasm of various species (mouse *vs.* porcine oocyte), so it had minority participation in the final epigenetic remodeling. The more significant component of the epigenetic remodeling is the oocyte cytoplasm (Barnetova et al., 2010). The role of oocyte quality is possibly also overestimated. Therefore there must be some other, already not defined, factors which affect the final epigenetic remodeling.

There is no doubt about the demethylation in mouse zygotes. However, mouse zygotes differ also in the time when the active demethylation occurs (Mayer et al., 2000; Santos et al., 2005). The differences in the time are possibly connected with mouse strains. We could expect such differences probably also in the pig. In our study we used progressed stage of zygotes which excluded a possibility of active demethylation to occur in later stage of the zygote. The active DNA demethylation was observed in the miniature pig (Fulka et al., 2006). A complete absence of active demethylation could be in hybrid breeds. These breeds were, for example, used in our work or in the work of Deshmukh et al. (2011).

The iICSI is a powerful technique which could be used also in human medicine for sperm quality evaluation. After the injection of human sperm into the mouse oocyte, the paternal pronucleus is formed (Fulka et al., 2008). These interspecies zygotes can be used for karyotype analysis (Araki et al., 2005), individual chromosome detection, or for detection of DNA amplification/deletion (Fulka H., personal communication). Further, as the human zygote cannot be practically used for evaluation of epigenetic remodeling, the iICSI enables us to test human sperm remodeling capabilities separately. In contrast to mouse oocytes, which were used for human sperm injection, the porcine oocytes are easy to obtain in quantity and the remodeling ability of commonly used *in vitro* matured oocytes is comparable to naturally ovulated oocytes. However, the possible use of porcine oocytes for the analysis in human reproduction must be tested furthermore.

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