—Original Article—

Epigenetic Characteristics of Paternal Chromatin in Interspecies Zygotes

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Abstract. Both the sperm and oocyte are terminally differentiated cells, but within a very short post-fertilization period, their genomes are converted into a totipotent zygote. The process of this transformation has been studied in a number of mammals as well as in the pig, for which very inconsistent results have been published. To clarify these inconsistencies, we have used the interspecies intracytoplasmic sperm injection technique for embryo production and subsequent paternal genome remodeling evaluation. First, we injected boar sperm heads into ovulated and *in vitro* matured mouse oocytes. The boar spermatozoa consistently decondense in ovulated oocytes and form fully developed pronuclei with demethylated DNA (5-methylcytosine; 5-MeC). Additional labeling against other histone modifications (H3/K9 dimethylation, H3/K4 trimethylation) and HP1 (Heterochromatin Protein 1) revealed similarity to those changes that are typical for natural mouse zygotes. On the other hand, no decondensation and formation of male pronuclei were observed, in spite of obvious oocyte activation, in *in vitro* matured oocytes. For this reason, we have evaluated the reprogramming parameters of *in vitro* matured mouse oocytes in more detail. In mouse zygotes (intraspecific), both pronuclei were consistently formed, but no sperm head chromatin demethylation was detected after 5-MeC labeling. Our observations suggest that porcine sperm heads are capable of undergoing active demethylation in *in vivo* matured mouse oocytes. On the other hand, *in vitro* matured oocytes possess much lower sperm remodeling capabilities.

Key words: Epigenetics, Interspecific, Mouse, Pig, Zygote

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The transformation of the sperm head into a male pronucleus represents a radical process during which highly compacted and condensed chromatin associated with protamines gradually decondenses, protamines are replaced with histones and certain epigenetic marks are removed [1]. Concomitantly, a new nuclear envelope is formed around the decondensing chromatin, and replication of DNA begins later on. This radical transformation converts the terminally differentiated state of the sperm into a paternal genome of a totipotent zygote. These processes are much less radical in the maternal genome. Thus, fertilization represents a major remodeling event that is accompanied by epigenetic changes of both parental genomes. Among the most prominent events studied so far is the global demethylation of paternal DNA [2–5]. It has been shown first in the mouse that levels of methylation differ between both pronuclei. Whilst the paternal pronucleus showed a negative signal after labeling against 5-methylcytosine (5-MeC), the maternal pronucleus remained heavily labeled [6, 7]. These processes were studied in some other species, and it has been suggested that this paternal genome demethylation step is a common phenomenon for all mammals [8]. However, some additional experiments have challenged this view. Thus, in the sheep and initially in the rabbit, both pronuclei showed the same level of

methylation, and much less extensive demethylation has been detected in bovine and human zygotes [4, 9, 10]. It was later shown even in the rabbit that the paternal genome does undergo the active demethylation step, leaving the sheep as the only exception reported so far [11]. Very inconsistent data has been published in the pig. In this regard, extensive demethylation of paternal DNA in *in vivo* produced embryos has been reported [8, 12]; on the other hand, the paternal genome was not demethylated in porcine zygotes when *in vitro* matured oocytes were used [13, 14]. It was later shown that the oocyte demethylation activity depends on the quality of oocyte maturation, namely on the period of germinal vesicle breakdown (GVBD) [13]. In those oocytes matured *in vivo* or where the oocytes were isolated from follicles after GVBD, paternal genome demethylation was observed consistently. On the other hand, in the majority of oocytes that were completely matured *in vitro*, no such prominent changes were detected [13].

To understand the processes of pig sperm head transformation in more detail, we have used interspecies intracytoplasmic sperm injection (iICSI), in which porcine sperm heads were injected into mouse oocytes matured either *in vivo* or *in vitro*. Interspecies ICSI allows us to separate experimentally the sperm and oocyte contribution to paternal genome remodeling after fertilization. Although this procedure does not completely reflect the intraspecies *in vivo* fertilization conditions, we believe that our approaches may shed indirectly some light on the processes of sperm head transformation and help to explain why contradictory results were obtained in some species.

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Fig. 1. Symmetrical and asymmetrical labeling in interspecies zygotes (porcine sperm injected into a mouse oocyte). (a) Labeling of interspecies zygotes against Pan-Histone revealed positive labeling in both pronuclei (sperm protamines were exchanged for oocyte histones). (b) Labeling with 5-MeC antibody revealed an extensive demethylation in the paternal pronucleus. (c and d) Labeling against HP1 also revealed the asymmetrical labeling. The paternal pronucleus is weakly labeled (c). Pronuclei after DAPI staining (d). MP, maternal pronucleus; PP, paternal pronucleus; PB, polar body.

Materials and Methods

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

Spermatozoa injection into in vivo or in vitro matured mouse oocytes

Porcine spermatozoa were obtained from a local insemination

station and kept in Solusem extender (AIM Worldwide) for up to one week. For ICSI, only highly motile spermatozoa were used. An aliquot of sperm suspension was washed several times in HTF (Cambrex, Verviers, Belgium), and a small volume (approximately 1000 sperm/1 μ l) of this suspension was transferred into a manipulation medium droplet. The selected spermatozoon was pressed by the injection pipette to the bottom of the manipulation Petri dish just between the sperm head and midpiece and rolled on its surface.

Fig. 2. Symmetrical and asymmetrical labeling in interspecies zygotes (porcine sperm injected into a mouse oocyte). (a and b) Labeling of interspecies zygotes against dimethyl H3/K9 revealed positive labeling in a maternal pronucleus and no labeling in a paternal pronucleus (a). Parallel DAPI staining (b). (c and d) Labeling against trimethyl H3/K4 revealed positive labeling in the maternal pronucleus and no labeling in the paternal pronucleus (c). Parallel DAPI staining (d). MP, maternal pronucleus; PP, paternal pronucleus; PB, polar body.

This resulted in separation of the sperm head from the tail. The isolated sperm head was then sucked into the injection pipette and injected into the oocyte cytoplasm, essentially as described by Kimura and Yanagimachi [15], on an inverted microscope stage (Olympus IX-70) with a PMM piezo injection system.

Mouse spermatozoa were stored in -70°C before use. After thawing, they were injected into metaphase II oocytes exactly as described by Kimura and Yanagimachi [15].

The ovulated oocytes were obtained from previously stimulated BDF1 mice with 7.5 I.U. of eCG (Calbiochem, San Diego, CA, USA) and 44 h later with 7.5 I.U. of hCG (Intervet, Boxmeer, Holland). The clusters of ovulated oocytes were isolated from oviductal ampulae approximately 14 h post-hCG and transferred into HTF with hyaluronidase (1 mg/ml) to dissolve the cumulus mass. The oocytes without cumulus cells were then washed several times in HTF and transferred into a manipulation droplet and

Table 1. Chromatin characteristics of paternal pronuclei in intraspecies and interspecies ICSI zygotes produced after sperm head injection into ovulated and *in vitro* matured mouse oocytes

Antibody	Intraspecies ICSI		Interspecies ICSI	
	Mouse sperm injected into mouse <i>in vitro</i> matured oocytes		Porcine sperm injected into mouse ovulated oocytes	
	MP	PP	MP	PP
Pan-Histone	+	+	+	+
5-MeC	+	+	+	–
HP1	+	–	+	–
Dimethyl H3/K9	+	–	+	–
Trimethyl H3/K4	+	–	+	–

MP, maternal pronucleus; PP, paternal pronucleus. +, positive labeling; –, weak/asymmetrical labeling.

Table 2. Chromatin characteristics of paternal pronuclei in intraspecies zygotes of the mouse and pig

Antibody	Mouse			Pig		
	MP	PP		MP	PP	
Pan-Histone	+	+	[20]	+	+	Barnetova, unpublished data
5-MeC	+	–	[8]	+	–	[8]
HP1	+	± ^{a)}	[18, 19]	+	+	Barnetova, unpublished data
Dimethyl H3/K9	+	–	[19]	+	± ^{b)}	[21]
Trimethyl H3/K4	+	± ^{a)}	[20]	+	+	[14]

MP, maternal pronucleus; PP, paternal pronucleus. +, positive labeling; –, weak/asymmetrical labeling; ±, different labeling in early and late zygotes. ^{a)} Antibody labeling appears in the advanced pronucleus. ^{b)} Variable antibody labeling, the pronucleus is sometimes labeled and sometimes not labeled.

injected with isolated sperm heads.

Mouse GV stage oocytes were isolated from large antral follicles of BDF1 mice stimulated previously with eCG (7.5 I.U., Calbiochem, San Diego, CA, USA). The oocytes released from follicles were carefully selected and then cultured in MEM supplemented with gentamicin (50 µg/ml), Na-pyruvate (0.22 mM) and BSA (4 mg/ml) for 12–14 h (5% CO₂ in air, 37°C). Then only those oocytes with first polar bodies were used for sperm head injection.

Culture of embryos and immunostaining

The injected oocytes were cultured for approximately 8–9 h in MEM supplemented with Na-pyruvate (0.22 mM), gentamicin (50 µg/ml) and BSA (4 mg/ml); thereafter, their zonae pellucidae were dissolved by pronase (0.5%). The oocytes were fixed in 4% paraformaldehyde and labeled with selected antibodies. The following antibodies were used for labeling: Pan-Histone (Roche, Prague, Czech Republic), anti-dimethyl H3/K9 (Upstate, Charlottesville, VA, USA), anti-HP1β (Abcam, Cambridge, UK), anti-trimethyl H3/K4 (Abcam) and anti-5-MeC (Eurogentec, Seraign, Belgium). The labeling and evaluation steps were essentially the same as described by Fulka *et al.* [16].

Results

In the first part of the study, we injected 251 ovulated mouse oocytes with pig spermatozoa, from which 178 (70%) survived the injection procedure. We have detected both pronuclei in all the oocytes. Whilst in porcine intraspecies one-cell stage embryos both pronuclei are approximately of the same size, in mouse intraspecies zygotes, the paternal pronuclei are much larger when compared with maternal pronuclei, which are typically located near second polar bodies. We observed a similar situation in the interspecies zygotes (mouse oocyte × pig sperm). When porcine spermatozoa were injected into mouse oocytes, the paternal pronucleus (porcine origin) was much larger than the maternal pronucleus (Figs. 1 and 2).

Labeling with anti-Pan-Histone antibody (32 zygotes) showed that the exchange of sperm head protamines for oocyte histones occurred in all cases (Fig. 1), and both pronuclei (maternal, paternal) were positively labeled. To further characterize the oocyte remodeling potential, we used several antibodies against different epigenetic marks and chromatin associated proteins. Labeling with the anti-5-MeC, anti-HP1, anti-dimethyl H3/K9 and anti-trimethyl H3/K4 antibodies clearly demonstrated the asymmetrical localization of these epigenetic marks; that is, the maternal pronuclei were positively labeled, whilst their counterparts (paternal pronuclei)

were negative or very weakly labeled (Figs. 1 and 2). This labeling pattern was typical for more than 75% of the evaluated zygotes; no labeling was typically detected in the rest of the zygotes. Thus, our results clearly show that the pig sperm chromatin responded to the mouse oocyte chromatin remodeling activities and essentially behaved as natural, i.e., mouse, sperm chromatin.

Because oocytes matured *in vitro* are almost exclusively used for *in vitro* fertilization and ICSI in the pig, in the next part of the study, we tested if the origin of oocytes influences the remodeling of paternal chromatin. In total, we injected 75 *in vitro* matured mouse oocytes with porcine sperm heads, and 69 (92%) of them survived the injection procedure. All these oocytes were activated because the second polar body was extruded. Surprisingly, no pronuclei were detected in about half of them (32) after 10 h in culture; the oocyte chromosomes were arranged as metaphase III chromosomes, and the sperm head remained intact or was slightly swollen (Hoechst staining). In the rest (37), the maternal chromatin decondensed and formed the female pronucleus, whilst the paternal chromatin remained intact or the sperm head only slightly enlarged. This did not allow us to use these zygotes for comparable immunolabeling.

As it has been clearly demonstrated that ICSI does not work well in the pig, we also tested if the sperm origin would influence the results of ICSI. We decided to test this with intraspecies ICSI (mouse sperm head injected into a mouse oocyte). In total, we injected 120 *in vitro* matured oocytes with mouse sperm heads (105 survived, and pronuclei were detected in 95 of them). When compared with *in vivo* oocytes, the only difference we observed was after 5-MeC labeling, where both pronuclei showed essentially the same intensity of fluorescence signal (25 zygotes). Labeling with the remaining antibodies did not differ from the *in vivo* controls (Tables 1 and 2).

Two essential conclusions arise from our study. First, the mouse ovulated oocyte cytoplasm efficiently induced decondensation of porcine sperm head chromatin, and fully developed pronuclei with prominent nucleoli were thereafter formed. However, a completely different situation was observed with *in vitro* matured oocytes, in which the porcine sperm heads did not respond to oocyte decondensation and remodeling factors. Moreover, in intraspecies ICSI (mouse $\times$ mouse), the *in vitro* matured oocyte cytoplasm is unable to induce efficient demethylation of paternal DNA.

Discussion

The iICSI technique is a powerful approach that allows us to experimentally divide the paternal and maternal contributions to genome remodeling in zygotes.

Maternal and paternal pronuclei are different in size in mouse intraspecies zygotes (the paternal pronucleus is much larger), and even after iICSI, we have observed that the paternal pronucleus of porcine origin is larger than the maternal pronucleus. This is somehow surprising because the sizes of both pronuclei are very similar in porcine zygotes (intraspecies). This indicates that pronuclear size regulation is a property of the oocyte—cytoplasm into which is the sperm introduced. Next, we evaluated the methylation pattern in interspecies zygotes and found no differences when compared

with mouse zygotes produced by intraspecies ICSI (mouse sperm heads injected into ovulated oocytes). This means that the mouse paternal genome undergoes active demethylation (5-MeC) in ovulated oocytes [17] and that the porcine male pronucleus is similarly demethylated in mouse ovulated oocytes. Furthermore, the mouse paternal genome is not methylated when other antibodies are used for labeling (dimethyl H3/K9, trimethyl H3/K4, HP1) [18–20]. Quite a different situation can be seen in porcine zygotes (intraspecies). Here, the parental genome in *in vivo* produced zygotes is actively demethylated (5-MeC) [8, 12]; on the other hand, this is not the case for *in vitro* produced zygotes [13, 14]. Contrary to the mouse, the porcine paternal genome also becomes methylated on dimethyl H3/K9 [21, Table 2].

Our results clearly show that the male porcine genome undergoes active demethylation in the mouse ovulated oocyte, although it is not demethylated in the porcine *in vitro* matured oocyte [14, 22]. This indicates that the absence of DNA demethylation in the porcine zygote is rather influenced by the quality of the oocyte.

To evaluate further the possible affect of oocyte quality on methylation pattern, we used *in vitro* matured mouse oocyte for iICSI. In the ovulated mouse oocytes used for iICSI, the sperm heads consistently activated the oocytes, and formation of the male pronuclei was quite efficient. On the other hand, when we used *in vitro* matured mouse oocytes, the porcine sperm heads did not form male pronuclei; in spite of that, the oocytes were activated. This means that the activation stimulus was not strong enough; that is, the second polar body was extruded, but the maternal pronucleus was not formed. Instead, the oocyte chromosomes were arranged as metaphase III stage chromosomes. The mouse *in vitro* matured oocyte therefore seems to have lower activation ability when compared with ovulated oocytes. The activation rate of *in vitro* matured oocytes is lower even in human-mouse iICSI zygotes [23]. Surprisingly, when mouse spermatozoa were injected into mouse *in vitro* matured oocytes, formation of both pronuclei occurred in almost all cases. Thus, the inability to activate the injected oocyte after ICSI may reflect some internal parameters of porcine spermatozoa [24].

To evaluate our presumption that the quality of *in vitro* matured oocytes could also affect the DNA methylation pattern, we used *in vitro* matured mouse oocytes for ICSI. The methylation pattern of these intraspecies mouse zygotes was different when compared with embryos originating from *in vivo* matured oocytes [17]. The paternal pronucleus was not actively demethylated, whilst active paternal genome demethylation is almost absolute in *in vivo* matured mouse oocyte cytoplasm [2–7]. Labeling with the remaining antibodies did not differ between both these groups, i.e., ovulated vs. *in vitro* matured oocytes.

Therefore, it seems that the activation capability and remodeling ability of *in vitro* and *in vivo* matured oocytes are not the same. When we compare the *in vitro* and *in vivo* matured oocytes in our experiment, it is clearly evident that the *in vivo* matured oocytes are of much higher quality and that they are able to induce efficient transformation of intra- or interspecies sperm heads into male pronuclei. Moreover, in interspecies *in vivo* oocytes, more efficient epigenetic modifications are detected when compared with *in vitro* matured oocytes.

In conclusion, our results support the notion that the DNA demethylation ability of the oocyte cytoplasm is influenced by a number of factors: the oocyte quality, culture conditions, labeling procedure and also by some intraspecies differences [25]. Nevertheless, even in this case, ICSI is a useful approach that may help us to clarify some controversial aspects associated with the demethylation phenomenon in zygotes. Moreover, this method can also be used in cases in which the oocytes of a given species are difficult to obtain [16].

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