The capability of reprogramming the male chromatin after fertilization is dependent on the quality of oocyte maturation

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Abstract

The present experiments compared the ability of pig oocytes matured either *in vivo* or *in vitro* to structurally reorganize the penetrated sperm chromatin into male pronucleus (PN) and to carry out, in parallel, the epigenetic processes of global chromatin methylation and acetylation, 12–14 h after *in vitro* fertilization (IVF). In addition, PN distribution of histone deacetylase (HDAC), a major enzyme interfacing DNA methylation and histone acetylation, was investigated. The ability of the oocyte to operate an efficient block to polyspermy was markedly affected by maturation. The monospermic fertilization rate was significantly higher for *in vivo* than for *in vitro* matured (IVM) oocytes ($P < 0.01$) which, furthermore, showed a reduced ability to transform the chromatin of penetrated sperm into male PN ($P < 0.01$). Indirect immunofluorescence analysis of global DNA methylation, histone acetylation and HDAC distribution (HDAC-1, -2 and -3), carried out in monospermic zygotes that reached the late PN stage, showed that IVM oocytes also had a reduced epigenetic competence. In fact, while in about 80% of *in vivo* matured and IVF oocytes the male PN underwent a process of active demethylation and showed a condition of histone H4 hyperacetylation, only 40% of IVM/IVF zygotes displayed a similar PN remodelling asymmetry. Oocytes that carried out the first part of maturation *in vivo* (up to germinal vesicle breakdown; GVBD) and then completed the process *in vitro*, displayed the same PN asymmetry as oocytes matured entirely *in vivo*. A crucial role of HDAC in the establishment of PN acetylation asymmetry seems to be confirmed by the use of HDAC inhibitors as well as by the abnormal distribution of the enzyme between the two PN in IVM zygotes. Collectively, these data demonstrated that some pig IVM oocytes fail to acquire full remodelling competence which is independent from their ooplasmic ability to morphologically reorganize the sperm nucleus into PN.

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Introduction

Many of the events that prepare the female gamete for fertilization and make it capable of supporting the initiation of embryonic development take place during oocyte maturation. Among these, cortical granule migration from subcortical to cortical regions (Cran & Cheng 1985, Galeati *et al.* 1991) in order to operate an efficient exocytosis (Ducibella *et al.* 1990) at fertilization, the development of clusters of endoplasmic reticulum in the oocyte cortex (Mehlmann *et al.* 1995), the increase of calcium in intracellular stores (Mehlmann & Kline 1994) to allow a prompt activation in response to fertilization (Carroll *et al.* 1994, Barboni & Mattioli 1996) and the increase of cytosolic glutathione levels for the decondensation of penetrated sperm nucleus (Mattioli *et al.* 1996, Sutovsky & Schatten 1997, Zuelke *et al.* 2003) are all essential steps

for the acquisition of full developmental competence. More recently, it has been found that the parental chromatin remodelling that occurs before singamy appears to be the most sophisticated and complex task that the oocyte has to accomplish in order for embryo/fetal development to proceed regularly (McLay & Clarke 2003).

The sperm nucleus, in fact, has a unique chromatin structure where the DNA is highly condensed, being associated with specific proteins, the protamines. This extremely packed chromatin organization is functional for the sperm, allowing a reduced cell size and a cell rigidity that are essential for penetration into the oocyte. However, in this condition, the sperm is associated with a reversible quiescence of the genome; there is no active RNA transcription or DNA replication (Ward & Coffey 1991, Ward & Zalensky 1996, Kramer & Kravetz 1997). Therefore, once it has entered the oocyte, sperm chromatin has to be

profoundly rearranged and made compatible with the maternal chromatin (Adenot *et al.* 1991, McLay & Clarke 2003). Following sperm penetration into the cytoplasm of a mature oocyte, the highly condensed chromatin of the sperm nucleus first decondenses and the protamines are replaced by histones (Kopecny & Pavlok 1975), after a short period of chromatin recondensation (Borsuk & Manka 1988, Wright & Longo 1988), a final phase of decondensation and the formation of the interphase male pronucleus (PN), surrounded by a new organized pronuclear membrane, occurs. During this sperm chromatin structure reorganization, and particularly during protamine–histone replacement, profound DNA epigenetic modifications take place. In the same mature ooplasm and at the same time, the maternal genome is also modified and prepared for integration with the paternal genome.

DNA methylation and histone acetylation are the two major mechanisms adopted for introducing epigenetic modifications both in paternal and maternal genomes. Several recent reports indicate that methylation of CpG islands and acetylation of the nuclear core histones condition the level of gene expression (Sapienza *et al.* 1987, Wolfe & Matzke 1999, Reik *et al.* 2001). In the early hours after fertilization, before DNA replication in the zygote, when the nuclear envelope breaks down and paternal chromosomes can directly interact with maternal cytosolic factors, the mature oocyte has to be able to operate a cycle where methylation patterns are reprogrammed genome-wide, but at the same time the epigenetic asymmetry established during imprinting between maternal and paternal genomes, still based on DNA methylation, has to be maintained (Reik *et al.* 2001). The arrangement of the cytoplasmic machinery required to carry out the above genome remodelling is most likely completed during oocyte maturation, as suggested by the findings of McLay & Clarke (1997), when structural components as well as specific enzymes first appear within the oocyte.

Manipulative procedures, such as cloning, due to damages related to nuclear–cytoplasmic interactions, may alter the capability of the oocyte to correctly perform these epigenetic processes leading to abnormal embryo development (Niemann *et al.* 2002, McEvoy *et al.* 2003, Wrenzycki & Niemann 2003). Moreover, the loss of imprinted gene marks can easily occur when the process of oocyte maturation is also carried out *in vitro*, invariably resulting in the developmental failure of embryos or leading to defective fetal programming (Niemann & Wrenzycki 2000, Young *et al.* 2001). Despite the fact that more than 20 years have been dedicated to optimizing oocyte maturation *in vitro* in a number of species, *in vitro* matured (IVM) oocytes still have an overall developmental competence that is far from normal and the kind of developmental abnormalities that are more frequently recorded, such as the large offspring syndrome in ruminants, point to aberrant epigenetic changes as the probable underlying cause (Niemann & Wrenzycki 2000, Niemann *et al.* 2002).

The ability of the oocyte to carry out the correct genome remodelling after fertilization therefore represents a solid opportunity to characterize oocyte maturation. For this reason, the knowledge of epigenetic processes in mammalian zygotes has acquired a particular relevance and several studies have addressed this issue in recent years. Results so far obtained demonstrate that active male DNA demethylation, originally recorded in the mouse (Mayer *et al.* 2000), is generally conserved in mammals (Dean *et al.* 2001), even though some differences for sheep and rabbits have been reported (Beaujean *et al.* 2004a, 2004b, Shi *et al.* 2004, Young & Beaujean 2004), suggesting that genome-wide demethylation is not an obligatory requirement for epigenetic reprogramming. As far as the process of histone acetylation is concerned, the only indications reported deal with the mouse model (Worrad *et al.* 1995, Adenot *et al.* 1997).

The present research has been designed to correlate the ability of the pig oocyte to organize the sperm nucleus into the PN with the main epigenetic modifications (DNA methylation and histone acetylation) of maternal and paternal chromatin that occur in zygotes 12–14 h after *in vitro* fertilization (IVF), shortly before singamy. As reported by Laurincik *et al.* (1994), at this time-point zygotes obtained from pig oocytes matured *in vivo* or matured *in vitro* do not show developmental differences. Even though during the early hours (between 6 and 12 h) after fertilization, male PN formation is delayed in most IVM oocytes (zygotes present asynchronous PN development, as compared with zygotes obtained from *in vivo* matured oocytes that already present synchronized PN 8 h after fertilization), during the next hours IVM oocytes present an acceleration of male PN formation and consequently between 12 and 14 h after IVF the developmental differences are no longer relevant in respect to *in vivo* matured oocytes. After this time (and until 32 h after fertilization) both PN develop in a well-synchronized manner. Therefore, in all our experiments, we utilized zygotes obtained from *in vivo* matured and IVM oocytes 12–14 h after IVF which did not present developmental differences and were characterized by the presence of synchronized opposing PN.

In order to verify whether structural and biochemical remodelling of male and female PN chromatin may be affected by the quality of the process of maturation, we studied, in parallel, three groups of oocytes: (a) oocytes that completed the whole process of maturation under *in vitro* conditions, (b) oocytes that resumed meiosis *in vivo*, up to germinal vesicle breakdown (GVBD), and completed the process *in vitro* and (c) *in vivo* matured oocytes.

The data presented here showed that IVM/IVF zygotes were characterized by a low ability to sustain monospermic fertilization as well as to develop well-synchronized PN and, in parallel, presented a reduced epigenetic competence. In fact, a high percentage of IVM zygotes fail to achieve demethylated DNA and hyperacetylated histone

H4 of paternal origin, as recorded in oocytes that completed the process of maturation *in vivo*. This ability to organize the global remodelling of sperm chromatin at fertilization can be obtained when the first part of the process of maturation (up to GVBD) is carried out *in vivo* and involves, in part, the selective distribution of histone deacetylase (HDAC)-2 in parental PN.

Materials and Methods

Source of oocytes

In vivo matured oocytes

Sixty prepubertal Large White gilts, with an average weight of 90 kg, were stimulated with 1250 IU equine chorionic gonadotrophin (Folligon; Intervet, Boxmeer, The Netherlands) to induce follicular growth, followed, 60 h later, by 750 IU human chorionic gonadotrophin (hCG; Corulon; Intervet) to trigger oocyte maturation and induce ovulation. Thirty-eight hours after hCG treatment (Tattini & Mattioli 1988), animals were slaughtered to collect the ovaries. Follicles of 6–10 mm in diameter were isolated and mature cumulus oocyte complexes were incubated for 2 h *in vitro* in modified TCM 199 (Sigma, St Louis, MO, USA).

IVM oocytes

Ovaries were collected soon after slaughter from prepubertal gilts and transferred in saline solution to the laboratory within 1 h. Healthy antral follicles 4–5 mm in diameter, selected on the basis of their translucent appearance, good vascularization and regular granulosa, were isolated under a stereomicroscope in Dulbecco's phosphate-buffered saline (PBS) supplemented with 0.4% (w/v) bovine serum albumin (BSA) and 70 µg kanamycin/ml. After isolation, the follicles were opened, turned inside out and incubated as everted follicles in a humidified atmosphere at 38.5 °C with 5% CO₂ in air in TCM 199 supplemented with 10% (v/v) fetal calf serum (FCS), 1 mmol glutamine/l, 70 µg kanamycin/ml, 1 µg luteinizing hormone/ml and 1 µg follicle-stimulating hormone/ml (USDA-pLH-B1 and USDA-pFSH-B1, kindly provided by the National Pituitary Hormone Distribution Program, NIDDK-NIH, Bethesda, MD, USA) to carry out oocyte maturation, according to Mattioli *et al.* (1989).

In vivo/vitro matured oocytes

As pig oocyte maturation is comparatively quite a long process, we were interested in assessing which part of maturation is more relevant for the acquisition of chromatin remodelling competence. With this aim, synchronized gilts were slaughtered 24 h after hCG injection, at about the time of GVBD. Follicles of 6–10 mm in diameter were isolated and incubated as everted follicles (Mattioli *et al.* 1989) to further mature oocytes for 20 h under *in vitro* conditions.

IVF

Oocytes collected at the end of *in vivo*, *in vitro* and *in vivo/vitro* maturation were mechanically denuded in dissection medium containing 0.1% (w/v) hyaluronidase using a fine glass pipette and selected for the presence of a polar body. Mature oocytes were then incubated in a 50 µl drop of fertilization medium (TCM 199; 10% (v/v) FCS, 8.4 mmol glucose/l, 1.25 mmol Na-pyruvate/l, 5.45 mmol calcium lactate/l and 2 mmol caffeine/l) under mineral oil at 38.5 °C. Fresh semen from two boars of proven fertility was used for the IVF procedure. According to Barboni *et al.* (1995), for capacitation *in vitro* the semen was washed in PBS with 0.4% (w/v) BSA through two successive centrifugations at 800 g for 3 min, then diluted in fertilization medium with 10% (v/v) FCS at a final concentration of 2×10^8 spermatozoa/ml and finally incubated for 2 h at 38.5 °C in a 5% CO₂ atmosphere. After capacitation, a 50 µl sperm aliquot was introduced in the fertilization drop to obtain a final concentration of 2×10^6 spermatozoa/ml. We utilized this sperm concentration which was higher than the one generally used (1×10^5 spermatozoa/ml) in order to maximally reduce the interval of sperm penetration into oocytes by working with a shorter time of co-incubation (2 h), according to Coy *et al.* (1993). Such a high sperm concentration produced an elevated incidence of polyspermy and therefore a low percentage of monospermic zygotes; nevertheless, with these conditions we were able to synchronize the time of sperm penetration and therefore the beginning of fertilization in order to obtain the most similar kinetics of PN development among the zygotes.

After the co-incubation with spermatozoa the mature oocytes were transferred into fresh fertilization medium and incubated for a further 10–12 h to reach the PN stage.

In order to assess the potential role of HDAC in the regulation of histone acetylation, a specific inhibitor of deacetylase enzyme, trichostatin A (TSA; 330 nmol/l; Sigma Chemical Co.), was used. With this aim a small group of *in vivo* matured oocytes were exposed to TSA during the whole IVF interval.

At the end of incubation all oocytes were briefly incubated in Tyrode's solution and transferred into 0.1% (w/v) pronase to remove the zonae pellucida and then fixed in acetic acid/ethanol (1:3) for 34–48 h and stained with lacmoid (Sigma).

Evaluation of events after IVF

Lacmoid-stained IVF oocytes were observed under a phase-contrast microscope in order to evaluate the fertilization rate, the percentage of monospermic oocytes and their ability to develop male PN. Oocytes were classified as fertilized when one or more spermatozoa had entered the oocyte and monospermic when a single sperm head (condensed or decondensed) or one male PN (identified by the sperm tail remnant nearby) was present within the ooplasm. Monospermic oocytes were classified in different developmental categories (PN1 to PN5) according to Laurincik *et al.*

(1994). In category PN1 the oocytes are completely penetrated by the sperm and the second meiotic division has resumed; in category PN2 the decondensation of chromosomes starts, the sperm tail is detached and the second polar body is extruded; in category PN3 the oocytes are characterized by further decondensation and by the appearance of the nuclear envelope. All these categories of oocytes were discarded and slides with monospermic oocytes belonging to category PN4–PN5, which are zygotes presenting spherical male and female PN with a complete nuclear envelope and maximum size close together, were selected and further analyzed for DNA methylation, histone H4 acetylation and HDAC distribution. Monospermic oocytes and the relative position of male and female PN were schematically noted and marked on each slide at the end of the morphological evaluation in order to identify, during the subsequent immunofluorescence analysis, the position of male PN.

Immunofluorescence protocol

Indirect immunofluorescence was carried out to evaluate and compare the pattern of DNA methylation, histone H4 acetylation and the subcellular localization of HDAC-1, HDAC-2 and HDAC-3 in PN4–PN5 zygotes obtained from *in vivo*, *in vitro* or *in vivo/vitro* matured oocytes.

With this aim the slides containing marked zygotes were washed with 0.05% (v/v) Tween-20 in PBS, fixed again for 2 h in 4% (w/v) paraformaldehyde in PBS and permeabilized with 0.2% (v/v) Triton X-100 in PBS for 1 h at room temperature.

For the detection of 5-methyl cytosine, the slides were further incubated for 2 h with 2 mol HCl/l at room temperature for DNA denaturation and subsequently neutralized for 20 min with 100 mmol Tris HCl/l buffer (pH 8.5). After washing with 0.5% (v/v) Tween-20 in PBS, the zygotes were blocked for 2 h at room temperature in 1% (w/v) BSA/0.05% (v/v) Tween-20/20% (v/v) goat serum and then incubated at 4°C overnight in the presence of a specific primary antibody (mouse monoclonal anti-5-methyl cytosine; Eurogentec, Seraing, Belgium) diluted 1:500 in 1% (w/v) BSA/0.05% (v/v) Tween-20 solution. The primary antibody was detected by a secondary antibody coupled with Cy3 (1:400 dilution; Molecular Probes, Eugene, OR, USA).

For the detection of hyperacetylated histone H4 and HDAC isoforms, the zygotes were incubated with the anti-hyperacetylated histone H4 (rabbit polyclonal, 1:300 dilution; Upstate Biotechnology, New York, NY, USA), anti-HDAC-1, anti-HDAC-2 and anti-HDAC-3 (rabbit polyclonal, 1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) primary antibodies. Anti-rabbit gamma globulin-Cy3 coupled (1:400 dilution; Molecular Probes) was then used as secondary antibodies. In experiments carried out on zygotes obtained from *in vivo* matured oocytes, negative control samples, in which the primary antibody was omitted, were also evaluated.

DNA was finally stained with 1 µmol SYBR 14/l (Molecular Probes).

Laser scanning confocal microscopy

Immunostained zygotes were observed with a Bio-Rad (Hercules, CA, USA) laser scanning confocal microscope (Radiance 2000 IK-2), equipped with a krypton/argon ion laser. The system was analyzed on an inverted microscope (Zeiss Axiovert) equipped with a plan-apochromat oil immersion objective 63× magnification /1.4 numerical aperture. The analysis of immunostained and DNA-labelled zygotes was simultaneously carried out by using the visible lines of excitation of 488 nm and 568 nm respectively, and a dichroic filter (560LP; Bio Rad). Digital optical sections were obtained by scanning a z-series of 0.2 µm slices throughout the planes of focus containing maternal and paternal PN; the sections were later projected to obtain a three-dimensional reconstruction and all the images were exported as 8 bit-tagged image file format files.

According to Beaujean *et al.* (2004b, 2004c), for quantitative measurements of the fluorescence emitted by each PN we merged the z-series collected with the LaserSharp software (Bio-Rad) to produce a two-dimensional image showing the staining pattern and the total fluorescence intensity of the two PN of the zygote analysed. The correction from background was obtained by subtracting the mean intensity of the cytoplasmic area to the whole image. Each PN was outlined manually in order to measure PN fluorescence intensity; the total fluorescence intensity emitted from each PN was measured using LaserPix software (Bio-Rad) and the values compared between male and female PN.

Statistical analysis

All experiments comprised six replicates involving a total of 348 *in vivo* matured oocytes, 564 IVM and 229 *in vivo/vitro* matured oocytes. All data were tested for normal distribution and then were analyzed by the Chi Square test and were considered significant at $P < 0.01$.

Results

Sperm penetration after IVF of oocytes matured *in vivo*, *in vitro* or *in vivo/vitro*

Fertilization rate after IVF, expressed as the percentage of oocytes that were penetrated by at least one sperm, was not affected by maturation with values of 62% ($n = 348$), 59% ($n = 564$) and 56% ($n = 229$) for *in vivo* matured, IVM and *in vivo/vitro* matured oocytes respectively (Table 1). By contrast, *in vivo* (78%; $n = 240$) and *in vivo/vitro* oocytes (82%; $n = 128$) showed a higher percentage of monospermic fertilization than IVM oocytes (36%, $n = 333$; $P < 0.01$; Table 1). In addition, whereas the majority of monospermic zygotes obtained from *in vivo* (90%; $n = 187$) and *in vivo/vitro* matured oocytes (85%;

Table 1 Evaluation of the events of fertilization in pig oocytes matured *in vivo*, *in vitro* and *in vivo/vitro* after IVF.

Source of oocytes	Total no. of fertilized oocytes (normal zygotes/ total no. of oocytes)	Fertilized oocytes (% of total oocytes)	Monospermic oocytes (% of total oocytes)	PN4–PN5 zygotes (% of total oocytes)
<i>In vivo</i> matured oocytes	348 (48%) ^a	240 (62) ^a	187 (78%) ^a	168 (90%) ^a
<i>In vitro</i> matured oocytes	564 (12%) ^a	333 (59) ^a	120 (36) ^b	65 (54) ^b
<i>In vivo/vitro</i> matured oocytes	229 (39%) ^a	128 (56) ^a	105 (82) ^a	89 (85) ^a

Data with a different superscript within each column are significantly different ($P < 0.01$).

$n = 105$) developed the late PN stage (PN4–PN5) 12–14 h after IVF, the same stage of PN development was recorded only in 54% ($n = 120$; $P < 0.01$) of IVM oocytes (Table 1).

Whole DNA methylation in zygotes obtained from *in vivo*, *in vitro* or *in vivo/vitro* matured oocytes

The analysis of DNA global methylation patterns showed that, in the majority of PN4–PN5 zygotes derived from *in vivo* and *in vivo/vitro* matured oocytes, the two PN displayed different fluorescence intensity (Fig. 1B). In fact, 80% ($n = 25$) and 73% ($n = 26$) of the PN4–PN5 zygotes respectively showed a demethylated male PN that resulted in about 38% less fluorescent (77.66; s.d. = 22.90) than female ones (125.20; s.d. = 16.84). In the remaining zygotes, a similar fluorescence intensity (difference $< 10\%$) in the male (114.70; s.d. = 15.52) and female (123.78; s.d. = 20.21) PN was recorded (Fig. 1E).

PN4–PN5 zygotes derived from IVM oocytes showed male PN hypomethylated relative to female PN only in 40% of cells analyzed ($n = 20$; $P < 0.01$; Table 2), whereas in the remaining zygotes PN displayed similar levels of DNA methylation.

Histone H4 acetylation in zygotes obtained from *in vivo*, *in vitro* or *in vivo/vitro* matured oocytes

Male and female PN exhibited different histone acetylation levels in most of the PN4–PN5 zygotes derived from oocytes matured *in vivo*. Eighty-two per cent of these cells ($n = 35$; Table 2) presented, in fact, the male PN about 29% more acetylated than the female one (114.85; s.d. = 25.44 and 81.54; s.d. = 15.22 respectively). Besides the different fluorescence intensity, the comparative analysis of chromatin histone acetylation in the two PN by laser scanning confocal microscopy revealed a different pattern of distribution of fluorescent signal between PN. Clusters of acetylated chromatin were irregularly distributed throughout male PN, whereas fluorescence was more homogeneously spread throughout female PN (Fig. 2B). Maturation affects the ability of the oocyte to differentially acetylate male and female histones. Zygotes derived from IVM oocytes showed the male PN more acetylated than the female one only in 38% of the cases ($n = 26$; $P < 0.01$; Table 2). Moreover 28% of zygotes derived from IVM oocytes had a male PN with the acetylated chromatin polarized in peripheral nuclear districts (Fig. 2D) while the remaining showed a similar pattern of fluorescence distribution and similar levels (difference $< 10\%$) of histone acetylation in female (108.79; s.d. = 24.32) and male (115.53; s.d. = 16.36) PN (Fig. 2E).

The zygotes derived from *in vivo/vitro* matured oocytes had a satisfactory ability to remodel paternal chromatin since the male PN was hyperacetylated relative to the female one in 76% of cases ($n = 34$; Table 2), a percentage similar to that recorded in zygotes obtained from *in vivo* matured oocytes ($P > 0.01$). Moreover, the fluorescence pattern of hyperacetylated chromatin in male PN

Figure 1 Confocal laser scanning micrographs of whole DNA methylation in pig zygotes at 12–14 h after IVF. (a, b and c) Representative images of zygote at the PN4–PN5 stage produced from *in vivo* matured oocyte that shows a demethylated male PN. (a) Green fluorescence indicates cell DNA stained with SYBR 14. Scale bar represents 20 μm. (b) Methylated DNA in male and female PN immunostained with anti-5-methyl cytosine antibody and Cy3-conjugated anti-mouse IgG. Scale bar represents 20 μm. The arrowhead indicates the position of the demethylated male PN, as confirmed (c) by the presence of sperm tail nearby (arrow). Scale bar represents 10 μm. (d and e) Example of zygote produced from an IVM oocyte showing similar levels of DNA methylation in male and female PN. Scale bars represent 20 μm. (d) Visualization of pronuclear DNA stained by SYBR 14. (e) Anti-5-methyl cytosine immunofluorescence of methylated DNA in male and female PN.

reproduced that recorded in zygotes obtained in physiological conditions (data not shown).

Subcellular distribution of HDAC isoforms in zygotes produced from *in vivo*, *in vitro* or *in vivo/vitro* matured oocytes

Experiments were carried out to investigate the distribution of HDAC-1, HDAC-2 and HDAC-3 in the different groups of PN4–PN5 zygotes, in order to understand the reasons for the different patterns of acetylation/methylation recorded in male and female PN. Preliminary analysis of the enzyme localization carried out in zygotes derived from *in vivo* matured oocytes showed that HDAC-1 ($n = 15$; Fig. 3) and HDAC-3 ($n = 12$; data not shown) isoforms were exclusively localized in the cytoplasm, suggesting that these isoforms are not involved in chromatin remodelling at the PN zygote stage. By contrast, HDAC-2 was clearly localized within the PN (Fig. 4), thus suggesting a direct contribution of this isoform of the

enzyme to post-fertilization chromatin remodelling. Consistent with the recorded acetylation patterns, fluorescence intensity was not similar between the two PN (Fig. 4B) and, in 89% ($n = 38$) of the zygotes obtained from *in vivo* matured oocytes, the male PN displayed about 33% lower fluorescence intensity (79.41; s.d. = 18.83) than the female one (118.52; s.d. = 32.54). A similar asymmetric fluorescence between the two PN was also recorded in 87% ($n = 29$; $P > 0.01$) of zygotes obtained from *in vivo/vitro* matured oocytes, while only in 44% ($n = 19$; $P < 0.01$) of the zygotes obtained from IVM/IVF oocytes (Table 2). In all groups, the remaining zygotes displayed the two PN with similar fluorescence intensity (difference $< 10\%$).

Role of HDAC-2 in PN chromatin remodelling

Following TSA treatment during the IVF interval, 78% of zygotes that developed late PN stages (PN4–PN5, $n = 16$; $P < 0.01$) lost the asymmetric level of acetylation between male and female PN, and female PN presented values of

Table 2 DNA methylation, histone H4 acetylation and HDAC-2 distribution in PN4-PN5 zygotes obtained from pig oocytes matured *in vivo*, *in vitro*, or *in vivo/vitro*

Source of oocytes	No. (%) of zygotes with demethylated male PN	No. (%) of zygotes with hyperacetylated male PN	No. (%) of zygotes with lower level of HDAC-2 in male PN
<i>In vivo</i> matured oocytes	25 (8%) ^a	35 (82) ^a	38 (89) ^a
<i>In vitro</i> matured oocytes	20 (40%) ^b	26 (38%) ^b	19 (44) ^b
<i>In vivo/vitro</i> matured oocytes	26 (73%) ^a	34 (76) ^a	29 (87) ^a

Data with a different superscript within each column are significantly different ($P < 0.01$).

Figure 2 Pattern of histone H4 acetylation in pig zygotes at 12–14 h after IVF. (a, b and c) Representative images of zygote produced from *in vivo* matured oocyte at the PN4–PN5 stage that shows differential histone H4 acetylation between the two PN. Scale bars represent 10 μ m. (a) Visualization of PN DNA stained by SYBR 14. (b) Comparative analysis between parental PN by confocal laser microscopy highlights a difference in both fluorescence intensity and pattern of fluorescence distribution obtained by zygote immunostaining with anti-hyperacetylated histone H4 antibody and Cy3-conjugated anti-rabbit IgG. Male PN (arrowhead) is characterized by a higher level of acetylation than female PN and its acetylated chromatin is organized in clusters rather than homogeneously distributed within the PN as observed in the female one. (c) Visualization by phase contrast microscope of the sperm tail (arrow) close to male PN following staining with lacmoid. (d and e) Two examples of abnormal distribution of acetylated chromatin in zygotes produced from IVM oocytes. Scale bars represent 10 μ m. (d) Zygote showing male PN with acetylated chromatin polarized in peripheral districts. (e) Zygote showing male and female PN with similar levels of histone H4 acetylation.

fluorescence intensity similar to that recorded in male PN (difference of fluorescence intensity <10%; 117.85; s.d. = 33.45 and 122.54; s.d. = 27.04 respectively; Fig. 5). In the remaining zygotes, female PN maintained the condition of reduced chromatin histone acetylation as compared with male PN.

Discussion

The present paper addresses the issue of the developmental competence of mature oocytes in the pig by focussing on the sequential steps that transform the penetrated sperm into male chromatin structurally and molecularly rearranged for integrating with female chromatin and

Figure 4 Subcellular distribution of HDAC-2 in pig zygote at 12–14 h after IVF. (a, b and c) Representative images of zygote at the PN4–PN5 stage produced from *in vivo* matured oocyte that shows differential HDAC-2 levels between the two PN. Scale bars represent 10 μ m. (a) Visualization of PN DNA stained by SYBR 14. (b) Visualization of HDAC-2 in male and female PN following immunostaining with the anti-HDAC-2 antibody and Cy3-conjugated anti-rabbit IgG. This isoform of the enzyme is localized inside PN and male PN (arrowhead) is characterized by a lower level of fluorescence as compared with the female one. (c) Visualization by phase contrast microscope of the sperm tail (arrow) close to male PN following zygote staining with lacmoid.

Figure 3 Subcellular distribution of HDAC-1 in pig zygote produced from *in vivo* matured oocyte double stained with immunofluorescence (anti-HDAC-1 antibody and Cy3-conjugated anti-rabbit IgG) and SYBR 14. Scale bars represent 20 μ m. (a) HDAC-1 isoform is localized in the ooplasm of the zygote, outside the two PN. (b) Merged image that simultaneously visualizes PN DNA stained by SYBR 14 and HDAC-1 distribution.

Figure 5 Pattern of histone H4 acetylation in pig zygote obtained from an *in vivo* matured oocyte exposed to TSA during the IVF interval. (a) Visualization of PN DNA stained by SYBR 14. Scale bar represents 10 μ m. (b) Following the inactivation of deacetylating enzyme, male and female PN present similar levels of chromatin histone H4 acetylation and within the male PN the acetylated chromatin is homogeneously distributed instead of being organized in clusters. Scale bar represents 10 μ m.

supporting full embryo development. All these changes rely exclusively on oocyte resources and hence their analysis might be useful for assessing the degree of oocyte maturation. The strategy adopted to carry out this study involved comparison between oocytes matured under physiological conditions, which have presumably acquired all that is required for a proper sperm nucleus remodelling and IVM oocytes which, despite the satisfactory results so far obtained (Mattioli *et al.* 1989, Prather & Day 1998, Hunter 2000, Abeydeera 2001, Nagai 2001, Niemann & Rath 2001), are still unable to guarantee normal developmental competence. For this reason, IVM oocytes may be a useful model to reveal the crucial functions that are conditioned by the process of maturation (Mattioli 1992, Sun & Nagai 2003, Bjerregaard *et al.* 2004).

The results presented here have confirmed that the ability of the oocyte to form male PN is strictly related to the degree of maturation, as *in vivo* matured oocytes were able to support the sperm–PN transition in the majority of the cases, while such a transformation was completed only in a small proportion of IVM eggs. This finding is consistent with the results presented by other authors (Laurincik *et al.* 1994) and confirms our previous investigations (Mattioli *et al.* 1988a, 1998b) which pointed to male PN formation as a key parameter to test the goodness of IVM procedures. Moreover, the analysis of *in vitro* experiments suggests that the acquisition of sperm remodelling competence follows a stepwise pattern. In fact, sperm chromatin decondensation, a first preliminary phase of sperm remodelling involving the reduction of protamine disulfide bonds, represents a primary level of competence since only some of the eggs capable of decondensing penetrated sperm can later support its transformation into male PN (Mattioli *et al.* 1988a, 1998b, Sun & Nagai 2003). In addition, we have demonstrated, in keeping with this finding, that only some of the zygotes that reached the PN4–PN5 stage can, subsequently, operate the physiological epigenetic chromatin rearrangement of the paternal genome. The percentage of PN4–PN5 zygotes obtained *in vitro* that we analysed in

our experiments was lower compared with the percentage of PN produced *in vitro* by other authors, but this could be because of the IVF procedure utilized, where a high concentration of spermatozoa was used to fertilize within a short window of IVF in order to synchronize the beginning of the fertilization process. In contrast, the incidence of normospermic fertilization cannot be deduced from the percentage of IVM/IVF zygotes that reached the blastocyst stage *in vitro*, since clear evidence has demonstrated that, in the pig, a percentage of embryos reach this stage of development even following polyspermic fertilization (Han *et al.* 1999, Kikuchi 2004).

Under physiological conditions, DNA methylation and histone acetylation are asymmetrically arranged in male and female PN in the pig. The active demethylation of sperm genome observed during the formation of a functional PN appeared to be similar to that described originally in mouse zygotes (Mayer *et al.* 2000, Oswald *et al.* 2000, Santos *et al.* 2002) and, more recently, in rat, bovine (Dean *et al.* 2001) and human zygotes (Beaujean *et al.* 2004a), while histone hyperacetylation recorded in male PN before singamy seems to follow a different kinetic from that described in the mouse model (Adenot *et al.* 1997). Such a species difference in paternal chromatin rearrangement is not surprising, since male PN demethylation seems not to be an obligatory requirement at the zygote stage in sheep and rabbit (Beaujean *et al.* 2004b, Shi *et al.* 2004) and re-methylation occurring in a number of species at blastocyst stage was not recorded in sheep embryos (Young & Beaujean 2004).

Post-fertilization genome remodelling therefore does not follow a conserved programme and epigenetic remodelling patterns cannot be automatically extended from one species to others.

In general, both maternal and paternal DNA are heavily methylated at the time of fertilization but a few hours later the male PN has undergone a marked demethylation. This action, which does not involve female PN at all, is quite surprising as both maternal and paternal chromatin are embedded in the same intracellular environment, the ooplasm, but DNA demethylation operates selectively towards paternal chromatin. As no DNA replication occurs during early PN development, zygotes have provided the first strong evidence of the existence of an active demethylation (Dean *et al.* 2001). In this context, the persistence of genome imprinting marks (Sapienza *et al.* 1987), based on cytosine methylation, emphasizes the complexity and selectivity of this process and its potential influence on embryo/fetal development.

In keeping with the changes in DNA methylation, we have here described, in the pig, higher levels of histone H4 acetylation in male PN than in the female one, thus confirming the inverse relationship between DNA methylation and histone acetylation proposed by a number of authors (Cervoni & Szyf 2001, Geiman & Robertson 2002, Lusser 2002) as a model to interpret genome regulation.

The influence of maturation on the competence of the oocyte to carry out a proper epigenetic remodelling of parental chromatin becomes evident from the results obtained when PN methylation and acetylation were recorded in zygotes from *in vivo* matured oocytes in comparison with those recorded in zygotes from IVM oocytes. While, in fact, the asymmetry between male and female PN occurs in approximately 80% of *in vivo* matured and IVF oocytes, only 40% of IVM/IVF zygotes presented this, presumably, adequate chromatin rearrangement. At present, we do not know exactly what may be the functional significance of the absence of differential PN remodelling but it certainly represents a deviation from what occurs under physiological conditions. Moreover, we have here shown that the ability to impose genome-wide epigenetic changes can be guaranteed in the zygotes when the first phase of maturation is carried out *in vivo*, after which meiosis can be completed *in vitro* without compromising the ability to perform DNA methylation and histone acetylation during the post-fertilization interval. This finding, apart from the possible physiological implication for understanding the events that characterize the process of oocyte maturation (McLay & Clarke 1997), may have an important impact in assisted reproduction where IVM is assuming a growing relevance and the use of oocytes committed to mature *in vivo* could be a promising strategy.

Chromatin remodelling depends on the synthesis of specific enzymes, their activation and, finally, their nucleus–cytoplasm distribution. To focus this issue, bearing in mind that the presence of methyl residues on DNA attracts a family of proteins that in turn fix HDAC on the chromatin (Fuks *et al.* 2003) and that the regulation of HDAC activity involves its nucleus–cytoplasm distribution (De Ruijter *et al.* 2003), the intracellular HDAC location was investigated. The enzyme is present with different isoforms (De Ruijter *et al.* 2003) but their distribution indicates that isoform 2, localized inside the PN, is the form that contributes to genome remodelling, while other isoforms studied, essentially concentrated in the cytoplasm, do not appear to play a role in chromatin rearrangement at the time of early embryo development. Although present in both PN, the HDAC-2 isoform is more concentrated in female PN, thus providing a potentially convincing explanation for the lower histone acetylation recorded in this chromatin. Histone acetylation may result, however, from the action of two opposite enzymes, histone acetyl-transferase and HDAC, but the observation that TSA, a potent HDAC inhibitor, virtually eliminated PN acetylation asymmetry points to the deacetylating enzyme as the major factor responsible for the differential PN acetylation. When nucleus–cytoplasm distribution of HDAC was investigated in zygotes from IVM oocytes, where asymmetrical PN acetylation was recorded in a low proportion of cells, in parallel a similar low percentage of zygotes displayed the asymmetrical distribution of the enzyme between male and female PN. This data further

confirmed the major role that HDAC plays in modulating histone acetylation under physiological conditions.

Interestingly enough, a small proportion of zygotes obtained following *in vivo* maturation did not exhibit the PN asymmetry and male and female chromatin could reach singamy having similar levels of DNA methylation and histone H4 acetylation.

If remodelling competence is conditioned by the process of maturation, it is likely that whenever maturation occurs under abnormal conditions, such as those following hormonal hyperstimulation (Barton *et al.* 2001, Shi & Haaf 2002) or under general bad nutritional conditions (Wu *et al.* 2004), this functional competence may not be completely achieved, as we recorded *in vitro*, thus compromising embryo–fetal development.

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