

# Changes in histone H4 acetylation during *in vivo* versus *in vitro* maturation of equine oocytes

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**ABSTRACT:** Epigenetic modifications are established during gametogenesis and preimplantation embryonic development. Any disturbance of the normal natural environment during these critical phases could cause alterations of the epigenetic signature. Histone acetylation is an important epigenetic modification involved in the regulation of chromatin organization and gene expression. The present study was aimed to determine whether the proper establishment of post-translational histone H4 acetylation at lysine 8 (AcH4K8), 12 (AcH4K12) and 16 (AcH4K16) of equine oocytes is adversely affected during *in vitro* maturation (IVM) when compared with *in vivo* matured oocytes collected from naturally cycling mares not undergoing ovarian hyperstimulation. The acetylation patterns were investigated by means of indirect immunofluorescence staining with specific antibodies directed against the acetylated lysine residues. Our results indicate that the acetylation state of H4 is dependent on the chromatin configuration in immature germinal vesicle (GV) stage oocytes and it changes in a residue-specific manner along with the increase of chromatin condensation. In particular, the levels of AcH4K8 and AcH4K12 increased significantly, while AcH4K16 decreased significantly from the fibrillar to the condensed state of chromatin configuration within the GV. Moreover, during meiosis, K8 and K12 were substantially deacetylated without any differences between *in vivo* and *in vitro* conditions, while K16 displayed a strong acetylation in oocytes matured *in vivo*, and in contrast, it was markedly deacetylated following IVM. Although the functional meaning of residue-specific acetylation during oocyte differentiation and meiotic resumption needs further investigation, our results support the hypothesis that IVM conditions can adversely affect oocyte ability to regulate the epigenetic reprogramming, critical for successful meiosis and subsequent embryonic development.

**Key words:** oocyte meiosis / histone H4 acetylation / epigenetics / chromatin configuration / meiosis / animal model

## Introduction

Epigenetic modifications, established during gametogenesis and preimplantation embryonic development, are involved in the regulation of chromatin conformation and function at specific stages of oocyte and embryo development. These modifications comprise DNA methylation at cytosines and covalent modifications of the histone proteins, which are involved in monoallelic gene expression of the embryo (genomic imprinting) as well as global or site-specific chromatin remodeling (Reik *et al.*, 2001; Li, 2002; Morgan *et al.*, 2005; De La

Fuente, 2006; Reik, 2007; Ciccone and Chen, 2009; Dindot *et al.*, 2009; Kota and Feil, 2010).

Any disturbance of the normal natural environment during these critical phases could cause alterations of the epigenetic signature. A number of reports have described an association between disorders of the epigenetic reprogramming and assisted reproductive technologies (ARTs) that may result in embryo death or birth defects (Cox *et al.*, 2002; Ludwig *et al.*, 2005 and reviewed in: De Rycke *et al.*, 2002; Thompson *et al.*, 2002; Gosden *et al.*, 2003; Huntriss and Picton, 2008). Although the majority of these reports have considered

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the possible effects of ART on the control of genomic imprinting process, a possible effect on global chromatin remodeling has been hypothesized (van der Heijden et al., 2009; Qiao et al., 2010; Ciapa and Arnoult, 2011).

The complete array of ART procedures, starting from ovarian hormonal stimulation to uterine embryo transfer, could have a profound impact on the epigenetic state of individuals born following assisted reproduction in both human and farm animals. Several experimental-based studies reported that superovulation (Sato et al., 2007), oocyte *in vitro* growth and maturation (Kerjean et al., 2003; Imamura et al., 2005; Borghol et al., 2006; Wang et al., 2010) and gamete and embryo culture media (Doherty et al., 2000; Sagirkaya et al., 2006; Market-Velker et al., 2010) can potentially alter epigenetic reprogramming.

Histones are major carriers of epigenetic information (Felsenfeld and Groudine, 2003; Fischle et al., 2003; De La Fuente, 2006; Gu et al., 2010) and covalent modifications of the core histone amino-terminal tails, including acetylation, are implicated in the control of gene expression and play important roles in chromosome organization and segregation during meiosis (De La Fuente et al., 2004; Akiyama et al., 2006). Interestingly, recent reports suggest that histone H4 acetylation could be involved in the complex spatial and temporal control of large-scale chromatin remodeling that precedes the resumption of meiosis (De La Fuente et al., 2004; Kageyama et al., 2007), which is critical to confer the oocyte with meiotic and developmental competence (Zuccotti et al., 1998; De La Fuente, 2006; Lodde et al., 2007).

In the mice (Kim et al., 2003; Akiyama et al., 2004, 2006; De La Fuente et al., 2004; Spinaci et al., 2004; Fulka, 2008; Manosalva and Gonzalez, 2009), pigs (Endo et al., 2005, 2008; Wang et al., 2006a, b), cows (Maalouf et al., 2008; Racedo et al., 2009) and humans (van den Berg et al., 2011), the temporal changes of H4 acetylation at specific highly conserved lysine residues (K5, K8, K12 and K16) and global deacetylation by histone deacetylase (HDAC) activity during oocyte meiosis are required for orderly meiotic events and accurate segregation of chromosomes. Notably, the inhibition of histone deacetylation during female gamete meiosis induces aneuploidy and embryo death in mice (De La Fuente et al., 2004; Akiyama et al., 2006). However, the information about acetylation dynamics of histones at specific K residues during meiosis is mostly derived from studies in which *in vitro* maturation (IVM) and/or superovulation protocols were used. To the best of our knowledge, the comparison with naturally ovulated oocytes has been reported only in mice (Akiyama et al., 2006). However, this is pivotal for the proper assessment of the 'epigenetic risks' related to ART.

Obtaining naturally ovulated oocytes for research purposes is particularly difficult in humans, where the availability of biological material suitable for research purpose is limited by the fact that, usually, only 'surplus' oocytes (that are considered not suitable for fertilization or that are obtained by patients who wanted to fertilize only some of their oocytes) can be used. Consequently, these oocytes, though being invaluable source of information, are affected by a number of limitations. In this scenario, the use of appropriate animal-based experimental models is essential to properly assess the risk of ART, for pre-clinical studies and for the investigation of basic biological mechanisms. Horse has been indicated as an excellent model to study oocyte maturation mechanisms, as mares are monovulatory with a longer phase of follicular development than most species and

their follicular wave pattern is similar to that of women (Ginther et al., 2004).

The aim of the present study is to determine the possibility that *in vitro* oocyte maturation may interfere with the proper establishment of post-translational histone H4 modifications. For this purpose, we analyzed and compared the dynamic changes of histone H4 acetylation at lysine 8 (AcH4K8), 12 (AcH4K12) and 16 (AcH4K16) during *in vivo* and *in vitro* maturation of equine oocytes.

In our model, hCG was administered to precisely time ovulation and recovery of pre-ovulatory oocytes in adult and naturally cycling mares, not exposed to ovarian hyperstimulation in order to reduce the factors that could influence the oocyte epigenetic signature.

## Materials and Methods

All the procedures described herein were reviewed and approved by the INRA Animal Care and Use Committee, and were performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals. All the chemicals were purchased from Sigma-Aldrich (St. Quentin Fallavier, France or Milan, Italy) unless otherwise indicated.

### Oocyte collection

#### Recovery of *in vivo* matured oocytes

Equine *in vivo* matured oocytes were collected by transvaginal ultrasound-guided aspiration in standing mares as previously described (Duchamp et al., 1987; Mugnier et al., 2009). Only adult cyclic pony mares in good body condition were used. Ovarian activity was assessed daily by transrectal ultrasound scanning. When a pre-ovulatory follicle  $\geq 33$  mm was detected, the mares were injected with 1500 IU hCG (i.v., Chorulon<sup>®</sup>, Intervet, France) to induce ovulation (Duchamp et al., 1987). Transvaginal ultrasound-guided aspiration of the pre-ovulatory follicle was performed 35 h after hCG injection. The mares were sedated with detomidine (3–5  $\mu$ g/kg, 0.1 ml/animal, i.v., Medesedan<sup>®</sup>; VIRBAC Sante Animal, Carros, France), and 5 min later a second injection of detomidine (6–10  $\mu$ g/kg, 0.15 ml/animal i.v.) and butylscopolamine bromure (0.2–0.3 mg/kg, 15 ml/mare i.v., Estocelan<sup>®</sup>; Boehringer Ingelheim, Paris, France) was administered to relax the rectum. After follicular fluid aspiration, follicles were flushed with Dulbecco's modified phosphate-buffered saline (DPBS, Dulbecco 'A'; Unipath, Dardilly, France) containing 5 IU/ml heparin (Sanofi-Aventis, Paris, France) at 37°C. At the end of the collection, the mares received an antibiotic injection (4 000 000 IU streptomycin, 4 g procaine penicillin i.m., Intramycine<sup>®</sup>; Ceva, Libourne, France). The aspirated fluids were examined under a stereomicroscope for the recovery of cumulus-enclosed oocytes (CEOs).

#### Recovery of immature oocytes

Immature CEOs were collected from follicles  $\geq 5$  and  $\leq 25$  mm in diameter, which are those used for standard IVM procedures. CEOs were collected either by transvaginal ultrasound-guided aspiration, as already described, or from ovaries recovered from slaughtered mares during the breeding season, as previously described (Luciano et al., 2006). Briefly, ovaries were recovered at the abattoir (Macello Di Parma S.R.L., IT 218 M, Parma, Italy or Entreprise Gourault, Blois, France) from pubertal females subjected to routine veterinary inspection and in accordance with the specific health requirements. Ovaries were transported to the laboratory within 3 h in sterile saline maintained at 26°C. The tunica albuginea was removed and follicles  $\geq 5$  and  $\leq 25$  mm in diameter were aspirated with a 16-gauge needle mounted on an aspiration pump (COOK-IVF, Brisbane, QLD, Australia) with a vacuum pressure of  $-28$  mmHg. The ovaries were cut into thick sections with a scalpel,

and follicles present in the ovarian stroma were aspirated. Follicular fluids were diluted ~1:5 with tissue culture medium (TCM)199 buffered with 20 mM HEPES (TCM199-HEPES) and supplemented with 1790 IU/l heparin and 0.4% bovine serum albumin (BSA). The aspirated fluids were examined under a stereomicroscope for CEOs recovery.

#### IVM of oocytes

Immature CEOs collected from slaughtered ovaries or retrieved by transvaginal ultrasound-guided aspiration from non-pre-ovulatory follicles  $\geq 5$  and  $\leq 25$  mm in diameter were matured *in vitro* as previously described (Goudet *et al.*, 2000; Luciano *et al.*, 2006). Briefly, CEOs were washed three times in TCM199-HEPES supplemented with 1790 IU/l heparin and 0.4% BSA. Groups of 15–25 CEOs were cultured in four-well dishes (NUNC, VWR International, Milan, Italy) for 28 h in 500  $\mu$ l of TCM199 supplemented with 20% fetal calf serum (FCS; Gibco, Invitrogen, San Giuliano Milanese, Milan, Italy), 0.68 mM L-glutamine, 25 mM NaHCO<sub>3</sub>, 0.2 mM sodium pyruvate and 50 ng/ml epidermal growth factor in humidified air under 5% CO<sub>2</sub> at 38.5 °C.

In a set of experiments, the follicles were measured with a ruler, classified according to the diameter in follicles 5–10 and 11–25 mm and their oocytes collected and cultured separately.

### Immunofluorescence staining and laser scanning confocal microscopy

Dynamic changes in AcH4K8, I2 and I6 in germinal vesicle (GV) and metaphase II (MII) stage oocytes were analyzed by indirect immunofluorescence, using polyclonal anti-AcH4K8 or anti-AcH4K12 or anti-AcH4K16 antibody (Upstate Biotechnologies, Inc., Lake Placid, NY, USA). CEOs were mechanically denuded in 500  $\mu$ l of TCM199-HEPES supplemented with 5% FCS and the zona pellucida was removed using 0.2% pronase as previously described (Mugnier *et al.*, 2009). After being washed three times in DPBS containing 0.1% polyvinylalcohol (PBS-PVA), the oocytes were fixed in 4% paraformaldehyde in DPBS for 1 h at room temperature. The fixed oocytes were washed three times with PBS-PVA and permeabilized with 0.2% Triton-X 100 in DPBS containing 0.05% Tween 20 (PBS-Tween) for 30 min at room temperature. Non-specific binding was blocked by incubating the samples in 20% donkey serum, 1% BSA in PBS-Tween for 1 h at room temperature. The samples were then incubated overnight at 4 °C with one of the earlier-mentioned primary antibodies diluted 1:250 in PBS-Tween containing 1% BSA. In each experiment, negative controls were performed by omitting the primary antibody. After being washed three times in PBS-Tween at room temperature for 10 min each, the oocytes were incubated with tetramethylrhodamine isothiocyanate labeled donkey anti-rabbit antibody (dilution 1:100; Vector Laboratories, Inc., Burlingame, CA, USA). Samples were washed three times in PBS-Tween, and mounted on slides in the antifade medium Vecta Shield (Vector Laboratories Inc.) supplemented with 20  $\mu$ M YO-PRO-1 (Molecular Probes, Invitrogen by Life Technologies, Carlsbad, CA, USA) for DNA counterstaining.

Samples were analyzed using a confocal laser-scanning microscope (IX81 FV500; Olympus) with a 60X objective. Digital optical sections were obtained by scanning the samples on z-axis at 0.7  $\mu$ m of thickness throughout the whole chromatin, as evidenced by YO-PRO-1 staining. The z-series were then projected to obtain a three-dimensional image. Instrument settings were kept constant for each sample.

Quantification of the immunofluorescence signals of AcH4K8, AcH4K12 and AcH4K16 in GV and MII oocytes was carried out on digitalized images using NIH ImageJ 1.4 Software (Abramoff *et al.*, 2004) after background subtraction. Data are expressed as the ratio of the specific acetylated residue fluorescence intensity divided by the YO-PRO-1 fluorescence intensity.

### Statistical analysis

All the experiments were repeated three to five times. Where appropriate, data were analyzed by analysis of variance (ANOVA) followed by the Newman–Keuls multiple comparison test or by Fisher's exact test (Prism, GraphPad Software, La Jolla, CA, USA). Regardless of the statistical test,  $P < 0.05$  was considered significant.

## Results

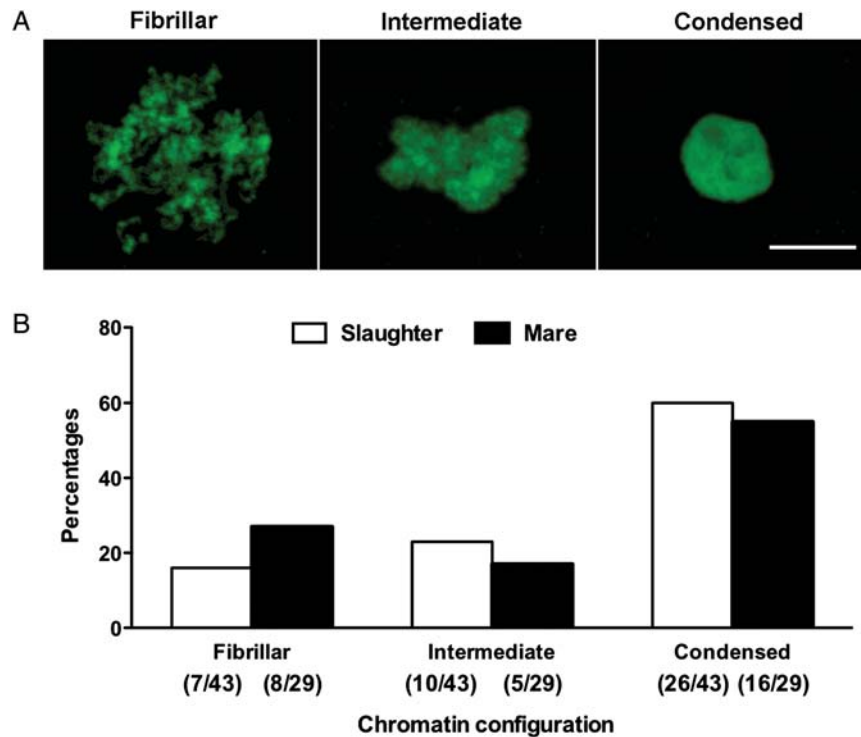
In this study, a total of 72 GV stage oocytes were evaluated, 43 of them were isolated from excised ovaries of slaughtered mares and 29 from non-pre-ovulatory follicles by ultrasound-guided aspiration in living animals. A total of 80 MII stage oocytes were analyzed, 43 of which were *in vitro* matured and 37 collected from pre-ovulatory follicles.

Preliminary experiments were conducted to precisely time the completion of maturation in our *in vitro* culture system and avoid *in vitro* aging of the MII stage oocytes. IVM time was tested in the range between 26 and 30 h of culture in three independent experiments. The 28-h culture period, in which the MII rate ranged between 67 and 83%, was chosen for all the following experiments. Oocytes that were not at the proper stage according to the time of isolation or culture (GV or MII) were excluded from the study.

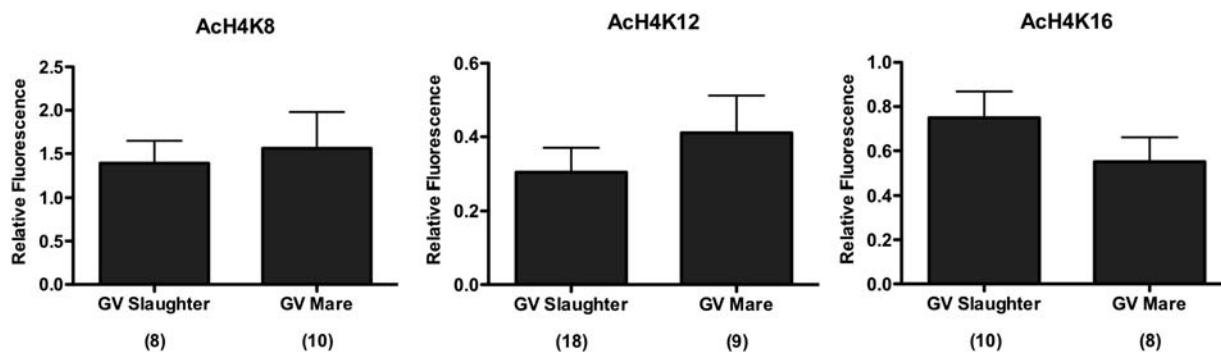
### Chromatin configuration and histone H4 acetylation in GV stage oocytes

After isolation, oocytes at GV stage were analyzed for chromatin organization according to the nuclear configuration as previously described (Hinrichs and Williams, 1997; Dell'Aquila *et al.*, 2001). As shown in Fig. 1A, three major classes of chromatin configuration, characterized by progressive condensation were observed: fibrillar, with diffuse filamentous chromatin; intermediate, with chromatin starting to form a single aggregate; condensed, with the chromatin condensed into one dense clump. The distribution of different chromatin configurations in GV stage oocytes is illustrated in Fig. 1B according to the retrieval method. Our results indicate that the condensed class was the most common (64 and 55% in slaughtered ovaries and mare, respectively). Moreover, the distribution of fibrillar, intermediate and condensed classes of chromatin configuration was not affected by the isolation method. As shown in Fig. 2, no changes in relative fluorescence intensity of H4K8, H4K12 or H4K16 acetylation in GV oocytes were observed according to the method of collection, as GV oocytes retrieved from excised ovaries had acetylation levels similar to those of oocytes collected from immature (i.e. not pre-ovulatory) follicles in living mares.

As shown in Fig. 3, the quantification analysis of the immunofluorescent signals revealed significant differences in the acetylation pattern of AcH4K8, AcH4K12 and AcH4K16 according to the GV chromatin configuration. In particular, our data indicated that the relative fluorescence intensity of AcH4K8 gradually decreased in relation to chromatin condensation. In fact, significant higher fluorescence intensity was detected in fibrillar GV than in oocytes with more condensed chromatin configuration ( $P < 0.05$ ). Similarly, also for AcH4K12, a significant decrease of fluorescence intensity was observed along with the progressive chromatin condensation ( $P < 0.05$ ). On the contrary, the analysis conducted on AcH4K16



**Figure 1** (A) Representative images of fibrillar, intermediate and condensed chromatin configuration at GV stage. (B) Distribution of fibrillar, intermediate and condensed GV stages in oocytes isolated from ovaries of slaughtered mares (Slaughter) and oocytes isolated by transvaginal ultrasound-guided aspiration (Mare). Values were analyzed by Fisher's exact test. No differences were observed between groups. Bar 10  $\mu$ m.



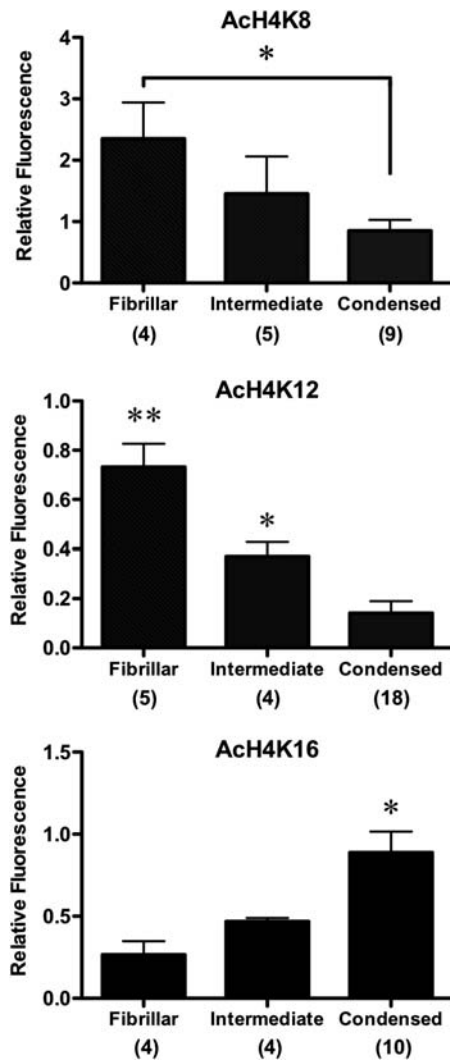
**Figure 2** Acetylation state of histone H4 at lysine 8 (AcH4K8), lysine 12 (AcH4K12) and lysine 16 (AcH4K16), immediately after isolation from ovaries of slaughtered mares (GV Slaughter) or by transvaginal ultrasound-guided aspiration (GV Mare). No differences were observed between groups. Oocytes were immunostained with specific antibodies against acetylated lysine 8, 12 and 16 on histone H4 (AcH4K8, AcH4K12 and AcH4K16) immediately after isolation (GV). Note that the Y-axis scale is different in each graph.

showed that the specific staining for this residue was significantly higher in condensed than fibrillar and intermediate GV stage oocytes ( $P < 0.05$ ).

### Histone H4 acetylation during *in vitro* and *in vivo* maturation

In this experiment, the changes in the immunofluorescence intensity of AcH4K8, AcH4K12 and AcH4K16 during *in vitro* and *in vivo* maturation

were compared. As shown in Fig. 4, the relative fluorescence of AcH4K8 and AcH4K12 is significantly lower in MII stage oocytes when compared with GV stage ( $P < 0.05$ ), with no differences between *in vitro* or *in vivo* maturation conditions. In contrast, the analysis conducted on H4K16 indicated that this residue was significantly deacetylated in MII oocytes after IVM, while *in vivo* matured oocytes displayed a significantly higher relative fluorescence ( $P < 0.05$ ), similar to that observed at GV stage.



**Figure 3** Histone acetylation in GV stage oocytes. Relative fluorescence of AcH4K8, AcH4K12 and AcH4K16 in fibrillar, intermediate and condensed GV stage oocytes. Data were analyzed by ANOVA followed by the Newman–Keuls multiple comparison test (\*, \*\* $P < 0.05$ ). Note that the Y-axis scale is different in each graph.

To give further insight into the process of acetylation/deacetylation during oocyte meiosis, we compared the fluorescence intensity of GV stage oocytes with condensed chromatin, which are those characterized by a higher meiotic competence (Hinrichs and Williams, 1997) with that of MII stage oocytes, matured *in vivo* or *in vitro*. Interestingly, this analysis revealed that, acetylation at K8 decreases even further from the GV-condensed oocytes to the MII stage oocytes, matured either *in vivo* or *in vitro*, ( $P < 0.05$ , Supplementary data, Fig. S1A). Accordingly, a decrease of AcH4K12 fluorescence intensity mean was found between condensed GV and *in vivo* or *in vitro* matured oocytes (Supplementary data, Fig. S1B). This difference was not statistically significant though, indicating that major deacetylation events at H4K12 are already set in place before the GV break down. Finally, the mean AcH4K16 fluorescence intensity of condensed GV did not differ from that of MII *in vivo* matured oocytes, while it decreased significantly in

*in vitro* matured oocytes (Supplementary data, Fig. S1C), confirming data obtained comparing AcH4K16 levels in total GV oocytes versus *in vivo* and *in vitro* matured oocytes (Fig. 4).

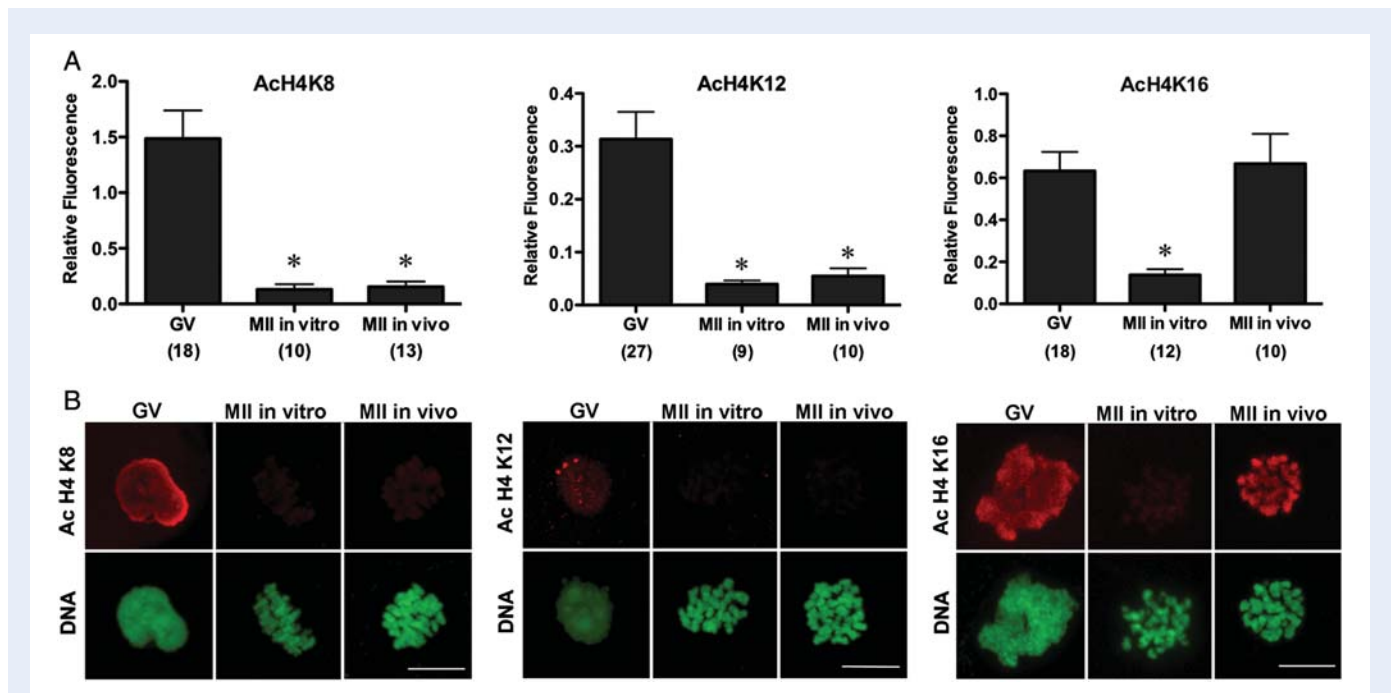
In order to further test the hypothesis that the alterations observed in H4K16 acetylation dynamics in *in vitro* matured oocytes versus the *in vivo* matured ones were mainly due to IVM procedures rather than other factors, such as the oocyte origin, we retrieved oocytes from follicles of different size (5–10 mm or 11–25 mm), matured them *in vitro* and compared the relative fluorescence of AcH4K16 with that of *in vivo* matured oocytes. As shown in Fig. 5, no significant differences were observed in the relative fluorescence of AcH4K16 between *in vitro* matured oocytes collected either from 5 to 10 or from 11 to 25 mm follicles. Importantly, H4K16 was always significantly more acetylated in *in vivo* matured oocytes than *in vitro* matured ones, even when different follicular sizes were considered.

## Discussion

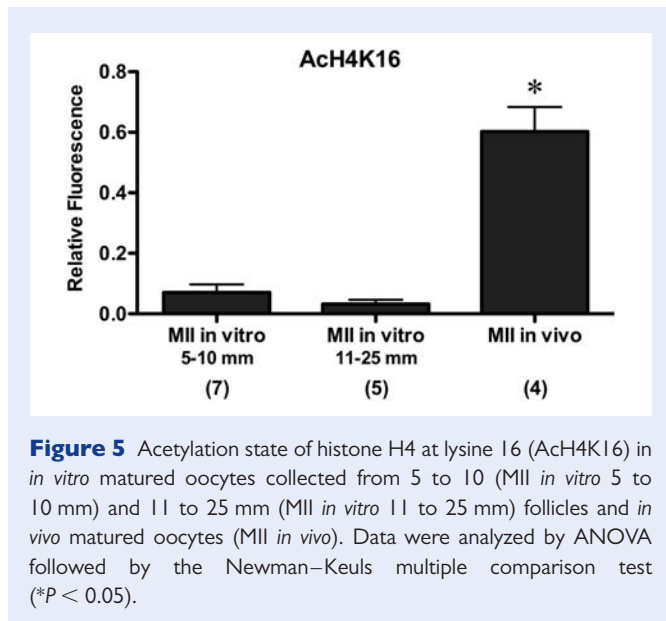
The present study indicates that IVM conditions affect the pattern of H4 acetylation in a residue-specific manner when compared with *in vivo* matured oocytes in naturally cycling monovular species not undergoing ovarian hyperstimulation. In particular, our data show that, while H4K8 and H4K12 undergo a deacetylation process during meiosis both *in vivo* and *in vitro*, the epigenetic remodeling at H4K16 is markedly affected in oocytes matured *in vitro*. MII chromatin, in fact, displays high levels of acetylation at H4K16 during naturally occurring meiosis while acetylation is significantly reduced in oocytes matured *in vitro*. Although additional factors, such as oocytes origin may contribute to the alterations observed in H4K16 acetylation dynamics during meiosis, our data indicate that the contribution of IVM is critical. This is confirmed by the fact that oocytes collected either from 5 to 10 or from 11 to 25 mm follicles and cultured *in vitro* to the MII stage showed the same pattern of acetylation at H4K16, and both were significantly deacetylated than *in vivo* matured oocytes, suggesting that IVM exerts a similar effect on oocytes retrieved from follicles of different sizes.

The disruption of H4K16 acetylation in oocytes matured *in vitro* could potentially affect the subsequent embryo development by perturbing chromosome segregation and alignment, as it has been demonstrated for misregulation of H4K12 acetylation in mouse (Akiyama *et al.*, 2006) and human (van den Berg *et al.*, 2011); however, this hypothesis remains to be investigated.

Global histone deacetylation during meiotic progression has been described in several animal models such as mouse (Kim *et al.*, 2003; Akiyama *et al.*, 2004, 2006; De La Fuente *et al.*, 2004; Spinaci *et al.*, 2004; Huang *et al.*, 2007; Ola *et al.*, 2007; Manosalva and Gonzalez, 2009), pig (Endo *et al.*, 2005; Wang *et al.*, 2006a, b), sheep (Tang *et al.*, 2007) and cow (Maalouf *et al.*, 2008; Racedo *et al.*, 2009) as well as in humans (van den Berg *et al.*, 2011). The chromatin-wide deacetylation process during meiotic maturation is thought to be involved in the assembly of meiotic chromosomes (Akiyama *et al.*, 2006; De La Fuente, 2006) and the erasure of cell memory, which may be required for the reprogramming of the oocyte genome (Schultz *et al.*, 1999; Nagashima *et al.*, 2007). However, these data are mainly derived from experiments conducted on oocytes after IVM and/or superovulation, which can perturb the natural epigenetic reprogramming (reviewed in: De Rycke *et al.*, 2002; Thompson *et al.*,



**Figure 4** (A) Acetylation changes of histone H4 at (a) lysine 8 (AcH4K8), (b) lysine 12 (AcH4K12) and (c) lysine 16 (AcH4K16), during oocyte *in vitro* and *in vivo* maturation. Data were analyzed by ANOVA followed by the Newman–Keuls multiple comparison test (\* $P < 0.001$ ). Note that the Y-axis scale is different in each graph. (B) Representative images of AcH4K8, AcH4K12 and AcH4K16 staining after isolation (GV) or after *in vitro* and *in vivo* maturation (MI I). Red: acetylated residues; Green: DNA. Bar 10  $\mu\text{m}$ .



**Figure 5** Acetylation state of histone H4 at lysine 16 (AcH4K16) in *in vitro* matured oocytes collected from 5 to 10 (MI I *in vitro* 5 to 10 mm) and 11 to 25 mm (MI I *in vitro* 11 to 25 mm) follicles and *in vivo* matured oocytes (MI I *in vivo*). Data were analyzed by ANOVA followed by the Newman–Keuls multiple comparison test (\* $P < 0.05$ ).

2002; Huntriss and Picton, 2008). Moreover, inter- and intra-specific differences in the acetylation/deacetylation dynamics for pigs and sheep have been reported (Vang et al., 2006a, b; Tang et al., 2007). Although species-specificity factors could explain such discrepancies, experimental conditions may be the major causes of these differences (De Rycke et al., 2002; Dupont et al., 2009; Gu et al., 2010).

In this perspective, our data are of particular importance since they describe the behavior of histone H4 acetylation in oocytes from

naturally cycling mares, in which the follicular growth phase and the process of oocyte meiotic resumption were not influenced by ovarian hyperstimulation. Although a treatment with hCG in our experiments was necessary in order to recover the pre-ovulatory oocyte, this treatment did not affect the acetylation state of the immature oocytes since K8, K12 and K16 of H4 had similar acetylation levels in GV stage oocytes collected from non-pre-ovulatory follicles in either hCG-treated mares or slaughtered mares. However, a potential influence on MI I stage oocytes cannot be completely excluded.

According to the general principle that histones are deacetylated during meiosis, and that high levels of chromatin (or chromosomes) condensation are generally associated with deacetylated histones, our results showed that acetylation of H4K8 and H4K12 starts to decrease while chromatin is condensing within the GV and further decreases thereafter, in condensed MI I chromosomes. On the contrary, acetylation of H4K16 increases along with the increase of chromatin condensation within the GV and further increases thereafter, in *in vivo* matured MI I chromosomes, while it decreases in *in vitro* matured ones. Importantly, this suggests that the process of H4 acetylation/deacetylation at K residues during meiosis may be differentially regulated depending on the specific K residue considered. The higher relative fluorescence for AcH4K16 (i.e. the lesser deacetylation) we observed on *in vivo* versus *in vitro* matured oocytes suggests that H4K16 escapes the process of deacetylation during meiosis *in vivo*. IVM conditions may alter the control of the meiotic acetylation/deacetylation process, resulting in a global deacetylation of H4.

Experimental evidence suggests that acetylation of individual histones as well as of particular lysine residues on the same histone may exert specific functional effects by initiating changes in chromatin

conformation or by regulating protein–histone interactions (Grunstein, 1997; Strahl and Allis, 2000). In somatic cells, acetylation of H4K12 and H4K16 is maintained in the M phase of mitosis, indicating a memory of gene expression state for daughter cells (Kruhlak *et al.*, 2001). Among the four K residues at the N-terminal tail of H4, K16 is the most frequently acetylated in eukaryotes (Turner and Fellows, 1989; Smith *et al.*, 2003), and is specifically targeted by an exclusive category of histone acetyl-transferases (HATs) such as males absent on the first (Rea *et al.*, 2007) as well as other HDACs, like the Sir2 family (Imai *et al.*, 2000; Vaquero *et al.*, 2007). On the basis of these data, even though the functional meaning of residue-specific acetylation and the mechanism(s) involved during oocyte meiosis needs further investigation, it can be hypothesized that IVM conditions may determine a misregulation of the specific HDACs–HATs pathway, that could be responsible for the shift in the acetylation pattern of H4K16, without affecting the other residues.

Similar to the findings in matured oocytes, the study conducted on immature GV stage oocytes indicates for the first time that in the horse, the acetylation state of H4 is residue-specific and is dependent on the chromatin configuration. In fact, the analysis of H4K8, H4K12 and H4K16 acetylation pattern revealed that the progressive chromatin rearrangement from fibrillar to condensed configuration is characterized by a decrease of acetylation at K8 and K12 residues and by an increase of acetylation at K16 residue. These data are of particular interest since the process of chromatin remodeling that precedes the resumption of meiosis has been recognized as a marker of oocyte final differentiation and associated with the acquisition of meiotic and developmental competences (De La Fuente, 2006) in several species, including the horse (Hinrichs and Williams, 1997; Hinrichs and Schmidt, 2000; Dell'Aquila *et al.*, 2001; Hinrichs *et al.*, 2005; Hinrichs, 2010). Histone acetylation has been recognized as one of the mechanisms implicated in GV large-scale chromatin remodeling (De La Fuente *et al.*, 2004; Kageyama *et al.*, 2007) and provides an important epigenetic mechanism for the developmental control of global gene expression (De La Fuente, 2006). In this context, more detailed information on the modifications that occur at each lysine residues of H4 would be helpful to better understand the mechanisms that regulate histone's acetylation dynamics as well as large-scale chromatin remodeling during oocyte growth and differentiation. Therefore, descriptive studies on the behavior of this epigenetic marker along with the oocyte differentiation is pivotal for the understanding of the mechanisms that regulate chromatin remodeling and function and that ultimately influence oocyte competence. Moreover, since any disturbance of the normal natural environment during this critical phase could cause alterations of epigenetic signature (Huntriss and Picton, 2008), our data can be useful to evaluate the epigenetic risks and safety of ARTs aimed to rescue immature oocytes.

Although *in vitro* oocyte maturation is a very attractive reproductive technology that can potentially reduce the cost and risks of ovarian hyperstimulation associated with the administration of exogenous gonadotrophins (Liu *et al.*, 2003; Edwards, 2007), culture conditions can adversely impact *in vitro* oocyte maturation in human by increasing the aneuploidy rate, as already reported (Requena *et al.*, 2009; Christopikou *et al.*, 2010; Xu *et al.*, 2010). Culture-induced effects on epigenetic regulation have been reported also in farm-animal-assisted reproduction, where the most striking example of alteration of the stability of genomic imprinting in the embryo is represented by the large

offspring syndrome with *in vitro* conceived ovine and bovine animals (Young *et al.*, 1998, 2001). Our results support and expand the hypothesis that IVM conditions can adversely affect oocyte ability to regulate its epigenetic reprogramming, also by altering global scale histone modifications and not just by perturbing genomic imprinting. Accordingly, Wang and collaborators found that the expression of enzymes that control histone acetylation, such as the histone acetyl-transferase GCN5 and HDAC1, is down-regulated during mouse IVM, with alterations that are still evident in the embryos at the 2-cell stage (Wang *et al.*, 2010).

Finally, it is important to consider that multiple factors could potentially interfere with histone modification processes and global chromatin remodeling. Notably, the length of *in vitro* culture, the composition and supplementation of culture medium, the oocyte origin as well as the maternal age could play important roles on the oocyte's ability to remodel its chromatin. In particular, studies in mice (Akiyama *et al.*, 2006; Suo *et al.*, 2010) and humans (van den Berg *et al.*, 2011) indicate that advanced maternal age negatively influences the deacetylation process of H4K12 during meiosis and that this is associated with chromosomes misalignment in MII stage oocytes. In our study, AcH4K12 was not influenced by IVM procedures indicating that different factors could preferentially affect one particular residue, or more in general one particular modification, rather than others. This hypothesis remains to be investigated and it would be interesting to assess whether maternal ageing affect the acetylation state of H4K12 during maturation of horse oocytes.

In conclusion, in many western countries, between 1 and 3% of annual births are achieved by assisted reproduction (Gosden *et al.*, 2003; Grace and Sinclair, 2009) and clinical protocols evolve and new technologies emerge. The development of animal models to assess the extent to which *in vitro* culture conditions interfere with epigenetic processes in mammalian oocytes is of paramount importance because it can provide valuable information to assess the epigenetic risks and safety of ART.

## Supplementary data

Supplementary data are available at <http://molehr.oxfordjournals.org/>.

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## Authors' roles

F.F. and V.L. were involved in study design, acquisition of data, analysis and interpretation of data and drafting of the manuscript. G.G., G.D., S.D. and C.D. performed the *in vivo* study. I.T. performed the *in vitro* study. G.G. and S.D. played a role in critically revising the manuscript. A.M.L. took part in conception and design, analysis and interpretation

of data, writing of manuscript and approval of final version to be published. All authors approved the final manuscript.

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## Conflict of interest

None declared.

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