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Developmental regulation of histone H3 methylation at lysine 4 in the porcine ovary

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Abstract

Follicular growth and oogenesis involve highly dynamic changes in morphogenesis, chromatin structure, and gene transcription. The tight coordination of these events leads to ovulation of a mature oocyte and formation of the luteal tissue necessary to regulate embryo implantation and development. This entire process is regulated by numerous endocrine and *in situ* mechanisms. The role of epigenetic mechanisms in folliculogenesis, such as the biochemical modification of the DNA packaging proteins, the histones, is not well understood. Our objective was to determine the cellular and follicular stage-specific patterns of histone H3 methylation at lysine 4 (K4) in porcine preovulatory follicles and during luteinization in pig ovaries. Ovary tissues were collected from slaughtered prepubertal and cyclic gilts at various stages of the estrous cycle, pregnancy, and from ovaries recovered from gonatropin-treated gilts at 0, 24, and 38 h post human chorionic gonadotropin (hCG) injection. Samples were fixed in 4% paraformaldehyde and processed for embedding in paraffin and sectioned using standard histological protocols. Immunofluorescent staining was performed on 3 μm thick sections. The immunostaining pattern of mono-, di-, and tri-methylated histone H3-K4 and lysine-specific demethylase 1 (LSD1, also known as KDM1 or AOF1) was assessed. Interestingly, H3-K4 mono-, di-, and tri-methylation in follicles of prepubertal gilts was specifically distributed and developmentally regulated. While granulosa cells of primary, secondary, and early antral follicles were negative for H3-K4 methylation those from large antral follicles showed a striking upregulation in the cells located in the proximity to the oocyte. Specifically, the cumulus oophorus displayed intense staining for H3-K4 methylation and signals were strongest in the granulosa cells in the inner two cell layers of the follicular wall. Although all oocytes from primary to large antral stage follicles were positive for H3-K4 mono-, di-, and tri-methylation, the patterns of distribution were altered through oocyte follicle development. H3-K4 methylation in granulosa cells was dramatically reduced as time to ovulation approached and was low to undetected at 38 h post hCG treatment. H3-K4 mono-, di-, and tri-methylation in large luteal cells increased as differentiation evolved but remained low in small luteal cells. Strikingly, LSD1 (KDM1) expression was found to be restricted to the corpus luteum. In summary, this study provides new information on histone H3-K4 methylation patterns in the oocyte and follicle during folliculogenesis, which suggests that these epigenetic markers serve an essential regulatory role during folliculogenesis.

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Introduction

The ovary is responsible for the generation of oocytes capable of undergoing fertilization and for the production of steroid hormones required to prepare for fertilization and pregnancy. The follicle is the primary structure of the ovary and is comprised of an oocyte encompassed by one or more layers of somatic cells. Recent advances in the study of folliculogenesis in mammals demonstrate a tightly regulated program of genomic, epigenetic, and hormonal events that direct survival, proliferation, and differentiation of somatic cells (DeManno *et al*. 1999, Ruiz-Cortes *et al*. 2005, Pepin *et al*. 2007). Despite the fact that *in vivo* and *in vitro* experiments and transgenic mouse models have revealed key mechanisms that regulate folliculogenesis (Pangas & Matzuk 2004, Skinner 2005), our understanding of the involvement of epigenetic processes is just emerging (LaVoie 2005). Follicular development in mammalian species is driven by endocrine signals, including estrogens and the gonadotropin and intra-ovarian regulators such as growth and differentiation factor 9 (GDF9; Su *et al*. 2004, Knight & Glister 2006). Progression from the primordial to preantral stage is, in part, regulated by oocyte-derived factors that include GDF9 and bone morphogenic protein (BMP15), and is

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not dependent on gonadotropin support (Matzuk *et al*. 2002). As the follicle develops to the early antral stage, it becomes responsive to gonadotropin and its development is also influenced by intra-ovarian regulators (Craig *et al*. 2007). Signaling between the oocyte and granulosa cells has emerged as a key regulator of the primary-to-secondary-to-antral follicle transition, and members of the transforming growth factor-β (TGF-β) family are required for these transitions (Pangas & Matzuk 2004). Estrogen and follicle-stimulating hormone (FSH) are critical to the formation of antral follicles (Britt *et al*. 2002, 2004), which fail to form in FSH receptor knockout mice (Dierich *et al*. 1998).

Eukaryotic DNA is packaged into chromatin formed by nucleosomes composed of a histone octamer (H2A, H2B, H3, and H4) wrapped by 147 bps of DNA. Epigenetic mechanisms regulate chromatin higher order and the access of gene regulatory factors to the DNA (Li *et al*. 2007). DNA methylation and histone modifications are important epigenetic mechanisms regulating gene expression, development, and are deregulated in some cancers (reviewed in Goldberg *et al*. 2007). Post-translational modification of the amino terminus of histones acts to control access of the transcriptional machinery to the DNA through recruitment of effector molecules, or can trigger gene silencing through recruitment of transcriptional repressors (Kouzarides 2007). Histone modifications associated with gene activation include histone H4 acetylation and histone H3 methylation at lysine 4 (H3-K4; Barski *et al*. 2007, Kouzarides 2007). Genome wide studies in eukaryotes localize H3-K4 methylation to gene regulatory regions (Santos-Rosa *et al*. 2002, Ng *et al*. 2003, Sims *et al*. 2003). More recently, in high-resolution profiling of the human genome, H3-K4 methylation was correlated with gene activation (Barski *et al*. 2007).

Prior to birth in the mammalian ovary, oocytes are arrested at prophase I of meiosis. Once the ovarian follicle undergoes activation, oocytes are maintained under meiotic arrest at the diplotene stage during postnatal development. At puberty and from each estrous cycle on, the gonadotropin surge of luteinizing hormone (LH) promotes meiotic resumption (Eppig 2001). Epigenetic imprints in the form of DNA methylation are established during oocyte growth and differentiation (Bourc'his *et al*. 2001, Lucifero *et al*. 2004). In addition, epigenetic mechanisms serve additional regulatory roles, such as in the large-scale changes to chromatin structure, and in the control of gene expression (Patterton & Wolffe 1996, Pepin *et al*. 2007). Dynamic changes in chromatin structure and function occur during oocyte growth in mammalian species, including the pig (McGaughey *et al*. 1979, Wiekowski *et al*. 1997, Endo *et al*. 2005, Kageyama *et al*. 2007). These alterations in chromatin structure are characterized by changes to the initially decondensed configuration termed non-surrounded nucleolus (NSN; Debey *et al*. 1993). In the pig, this configuration is referred to as germinal vesicle 0 (GV0) and consists of a nucleolus with diffuse filamentous chromatin distributed over the nuclear area, which is seen in most of the oocytes present in follicles smaller than 1 mm (Sun *et al*. 2004). With the progression of growth and differentiation, oocytes undergo a dramatic change in nuclear organization in which chromatin becomes progressively condensed, forming a heterochromatin rim surrounding the nucleolus, a configuration termed surrounded nucleolus (SN; Debey *et al*. 1993), or GV1–4 (depending on the condensation state of the chromatin) in the pig (Sun *et al*. 2004). Accompanying these alterations in chromatin structure are changes in transcription levels; oocytes showing the NSN configuration undergo high rates of transcription whereas those in the SN state display only a low level (Bouniol-Baly *et al*. 1999, Maddox-Hyttel *et al*. 2005, De La Fuente 2006).

In the porcine ovary following the preovulatory LH surge, the luteinization process begins with hyperanemia and enlargement, followed by expulsion of the ovum (Murphy *et al*. 2001). Following ovulation, under the influence of the gonadotropins, prolactin and insulin, granulosa and thecal cells differentiate. This process is accompanied by alterations in steroidogenesis whereby the thecal and granulosa cell participate in the synthesis and secretion of progesterone (Murphy *et al*. 2001). At this point, the roles of histone modification in the control of gene transcription are just beginning to be elucidated. For example, acetylation of histone H3 at lysine 9 has been revealed to increase transcription of Niemann-Pick C1 in porcine granulosa cells (Gevry *et al*. 2003) and progesterone synthesis capacity is facilitated by upregulation of cholesterol synthesis for which *STAR* is a key gene whose expression appears to be regulated in part by epigenetic mechanisms (Lazzaro *et al*. 2006). During luteinization, granulosa cells undergo acetylation of histone H3 and consequent transcriptional activation of the *STAR* gene (Christenson *et al*. 2001). Given the importance of epigenetic modifications in the control of gene expression and chromatin organization, the present study was undertaken to determine the cell and developmental-specific patterns of histone H3-K4 mono (m1)-, di (m2)-, and tri-methylation (m3) in the porcine ovary. Immunohistochemistry with highly specific antibodies was used to localize histone H3-K4 methylation during folliculogenesis and luteinization. In order to gain further insight into the hormonal and temporal regulation of H3-K4 methylation in somatic cells, ovarian follicles were examined from synchronized gilts at 0, 24, and 38 h post-hCG injection. Patterns of H3-K4 methylation described here suggest that these marks may be regulated by endocrine and paracrine factors and function in transcriptional gene regulation in somatic cells and in chromatin reorganization during oogenesis. In addition, the newly identified histone H3-K4 demethylase, lysine-specific demethylase (LSD1, also known as K-demethylase, KDM1) was found to be restricted to the corpus luteum.

Results

Dynamic histone H3 lysine 4 methylation in porcine folliculogenesis and oogenesis

Immunofluorescent analysis of prepubertal porcine ovaries using antibodies specific for either histone H3 mono-, di-, or tri-methylation, revealed a highly specific distribution that was developmentally regulated. Granulosa cells of primary, secondary, and tertiary (beginning of antral cavity formation) follicles were negative for H3-K4 mono-, di-, and tri-methylation (Fig. 1). In large antral follicles, there is a striking upregulation of H3-K4 mono-, di-, and tri-methylation in the granulosa cells in proximity to the oocyte (Fig. 2). The cumulus oophorus displays intense staining for H3-K4 mono-, di-, and tri-methylation and signals were strongest in the granulosa cells in the inner two cell layers of the follicular wall (Fig. 2). Thecal cells of antral follicles were also positive for H3-K4 mono-, di-, and tri-methylation (Fig. 2).

We examined the pattern of histone H3 lysine 4 methylation in oocytes within ovarian sections demonstrating that the oocytes from primary to antral stage follicles were positive for H3-K4 mono-, di-, and tri-methylation, and the patterns of distribution were altered through oocyte follicle development (Figs 1 and 2). In primary follicles, identifiable by the single flattened layer of granulosa cells, H3-K4 mono-methylation appears to be associated with euchromatic regions, as evidenced by its exclusion from heterochromatin identifiable by intense 4′,6-diamidino-2-phenylindole (DAPI) staining (Fig. 1A). H3-K4 mono-, di-, and tri-methylation all show a punctate distribution in filamentous chromatin (GV0) of growing oocytes (Fig. 1A–I). Following extensive chromatin remodeling during oocyte maturation, H3-K4 mono-, di-, and tri-methylation were also detected in GV1–4 of oocytes in antral follicles (Fig. 2F, L and R).

Approaching ovulation triggers loss of H3-K4 methylation during the luteinization process

The interval from the LH surge to ovulation in pigs is ~30±3 h (Soede *et al.* 1994). In gilts synchronized with equine chorionic gonadotropin (eCG) followed by hCG, ovulation usually occurs at 42±2 h after the hCG injection (Downey & Ainsworth 1980). To gain insight into the epigenetic events underlying periovulatory follicle development, we collected follicles from eCG–hCG-treated gilts at 0, 24, and 38 h post-hCG injection. Tissue sections were then examined for ovulatory effects on H3-K4 mono-, di-, and tri-methylation. Strikingly, as time to ovulation approached, H3-K4 methylation was reduced and then completely lost from granulosa cells of the follicular wall at 38 h post-injection ($P<0.05$; Fig. 3).

The pattern of methylation loss during early luteinization prompted us to study how histone H3-K4 lysine methylation may function in cell differentiation in the luteinization process. Samples were prepared from gilt ovaries collected at the early, mid, late, and regressing stages of the corpus luteum lifespan. Immunofluorescence

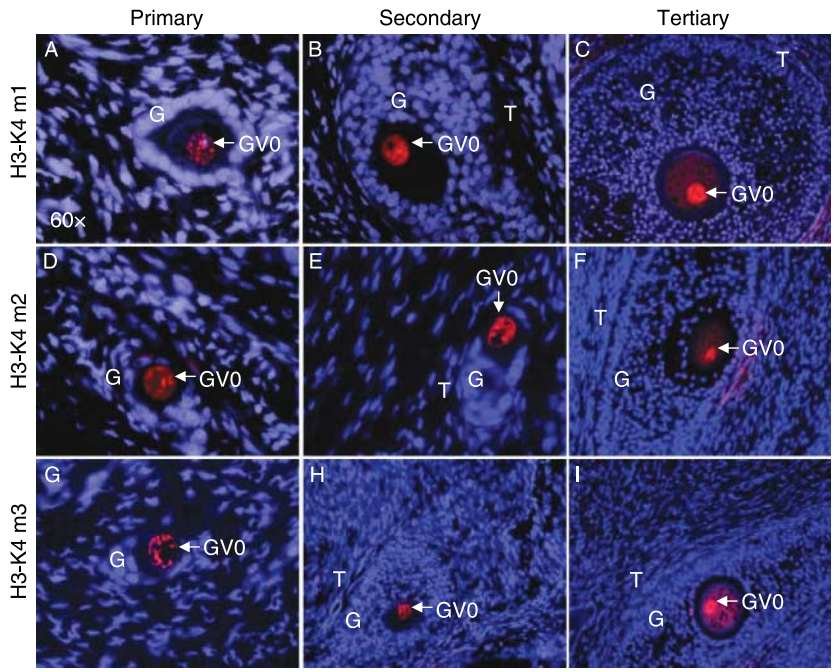

Figure 1 Immunofluorescent localization of methylation patterns of histone H3 at lysine 4 (H3-K4) in developing ovarian follicles. Immunofluorescence using antibodies specific for (A–C) H3-K4 mono-methylation (H3-K4-me1, red), (D–F) H3-K4 di-methylation (H3-K4-me2, red), and (G–I) H3-K4 tri-methylation (H3-K4-me3, red) shows an oocyte-restricted localization of methylated H3-K4. DAPI counter staining (blue), theca (T), granulosa (G), and non-surrounded nucleolus with diffuse filamentous chromatin distributed over the nuclear area (GV0). Arrows indicate that the nucleolus and all images are at 60× magnification.

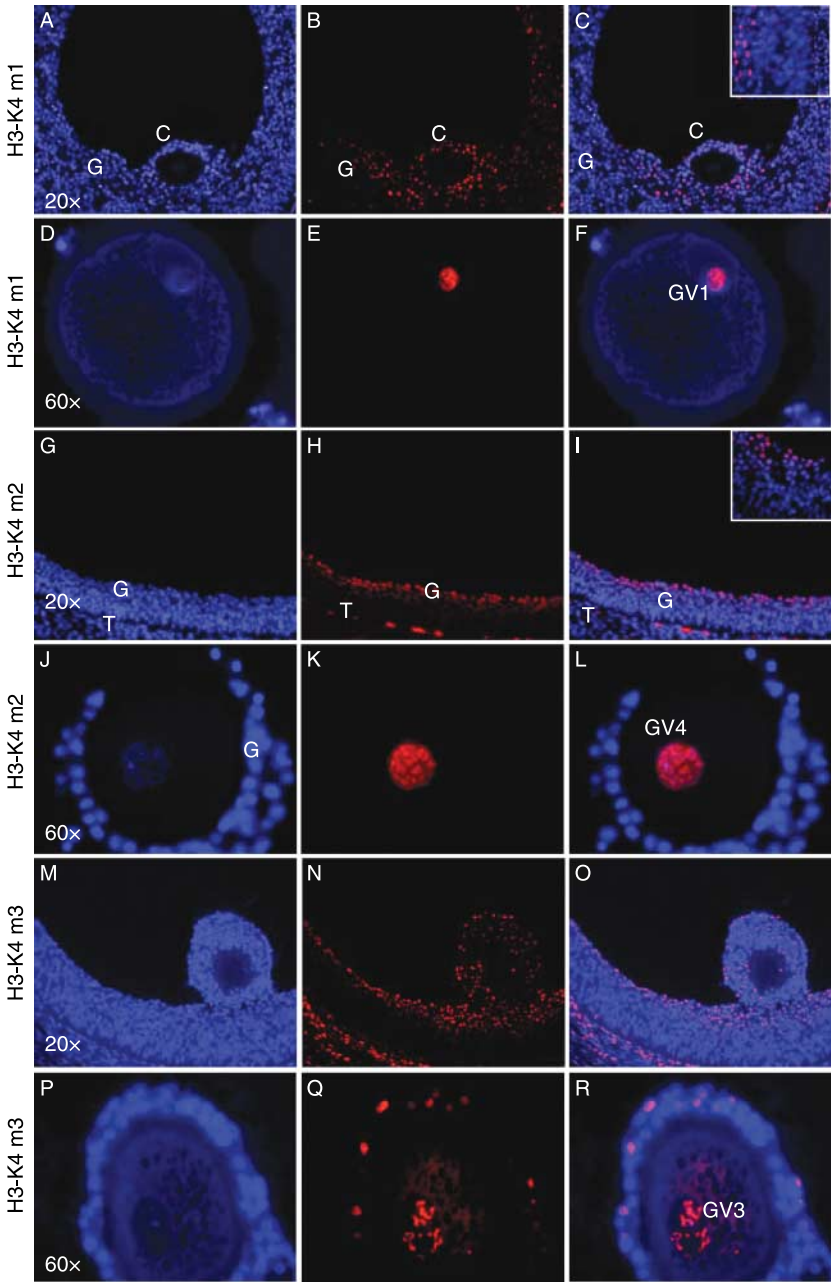

Figure 2 Immunofluorescent localization of methylation patterns of histone H3 at lysine 4 (H3-K4) in antral follicles. Immunofluorescence using antibodies specific for (A–F) H3-K4 mono-methylation (H3-K4-me1, red), (G–L) H3-K4 di-methylation (H3-K4-me2, red), and (M–R) H3-K4 tri-methylation (H3-K4-me3, red). DAPI counter staining (blue), theca (T), granulosa (G), cumulus (C), germinal vesicle configurations (GV1, GV3, and GV4). Magnification for each row of images is indicated.

analysis revealed increasing histone H3-K4 mono-, di-, and tri-methylation in large luteal cells as differentiation evolved (Fig. 4). All three signals were weak in small luteal cells. At the onset of luteal regression, signals for H3-K4 lysine 4 methylation were lost (Fig. 4). In pregnancy, strong methylation of H3-K4 methylation was present in large luteal cells.

LSD1 or KDM1 is restricted to the corpus luteum

LSD1 (KDM1) is specific for the reversal of mono- and di-methylation of H3-K4 (Shi *et al.* 2004, Lee *et al.* 2006*b*), and functions in concert with other chromatin-modifying proteins such as the histone deacetylases HDAC as a transcriptional repressor (Lee *et al.* 2006*a*). Given our observations (Figs 3 and 4) that H3-K4 lysine methylation appears to be regulated in folliculogenesis and in corpus luteum formation, we investigated the distribution of LSD1 (KDM1) in porcine ovaries. Remarkably, no LSD1 (KDM1) was detected in follicles (data not shown), while coinciding with maximal steroidgenesis and full differentiation, LSD1 (KDM1) abruptly appeared in large luteal cells from days 11 to 17 of the porcine estrous cycle and was sustained during pregnancy (Fig. 5). At regression, LSD1 (KDM1) was no longer detected.

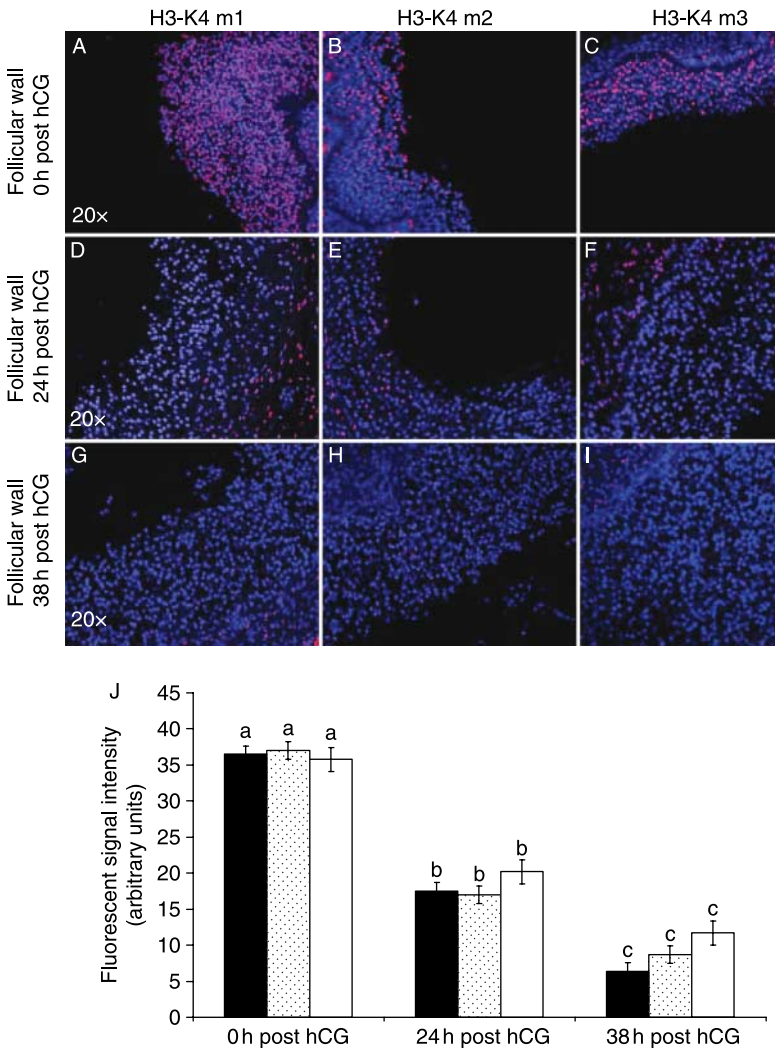

Figure 3 Methylation of histone H3 at lysine 4 (H3-K4) in follicular walls of equine chorionic gonadotropin–hCG-treated gilts decreases as follicular remodeling proceeds. H3-K4 m1, m2, and m3 methylation is shown at (A–C) 0-, (D–F) 24-, and (G–I) 38 h post hCG injection. DAPI counter staining (blue). (J) A graphical representation of fluorescent signal intensity is shown. Bars with different letters are significantly different ($P<0.05$).

Discussion

To date, relatively little information is available on the role of epigenetic processes regulating differentiation, signaling, and development in the ovary. In the present study, we provide the first information about the combined histone H3-K4 methylation patterns in the oocyte and follicle during folliculogenesis. In the oocyte and surrounding somatic cells, we observed dynamic patterns of histone H3-K4 methylation that implicate these histone modifications in chromatin remodeling and the regulation of transcription and differentiation. Methylation of histone H3-K4 is a dynamic gene-specific process (Barski *et al.* 2007), which is carried out by histone lysine methyltransferases, such as Meisetz (meiosis-induced factor containing PR/SET domain and zinc-finger motif; Hayashi *et al.* 2005), MLL (mixed-lineage leukemia), and SMYD3 (SET- and MYND domain-containing protein; Bannister & Kouzarides 2005). The recent discovery of several histone H3-K4 demethylases such as LSD1 (KDM1; Shi *et al.* 2004), has revealed a highly regulated coordination between methylation states in control of gene expression in response to hormonal cues, such as activation of the androgen receptor (Metzger *et al.* 2005). Investigation of paracrine and endocrine regulation of oogenesis and folliculogenesis has revealed oocyte and granulosa-specific regulatory pathways for meiosis and mitosis (Kezele & Skinner 2003, Britt *et al.* 2004, Knight & Glister 2006).

In a previous study we demonstrated epigenetic coordination of granulosa cell mitosis by Aurora-B kinase, a serine threonine kinase that phosphorylates histone H3 at serine 10 (H3-S10; Ruiz-Cortes *et al.* 2005). In the ovary, estrogen and FSH stimulate Aurora-B kinase activity presumably regulating granulosa cell proliferation (Ruiz-Cortes *et al.* 2005). Histone H3-S10 phosphorylation was present in proliferating granulosa cells of developing follicles from the primary to antral stage (Ruiz-Cortes *et al.* 2005). In contrast, H3-K4 methylation in granulosa cells was highly restricted. For all H3-K4 methyl marks examined, little or no reactivity

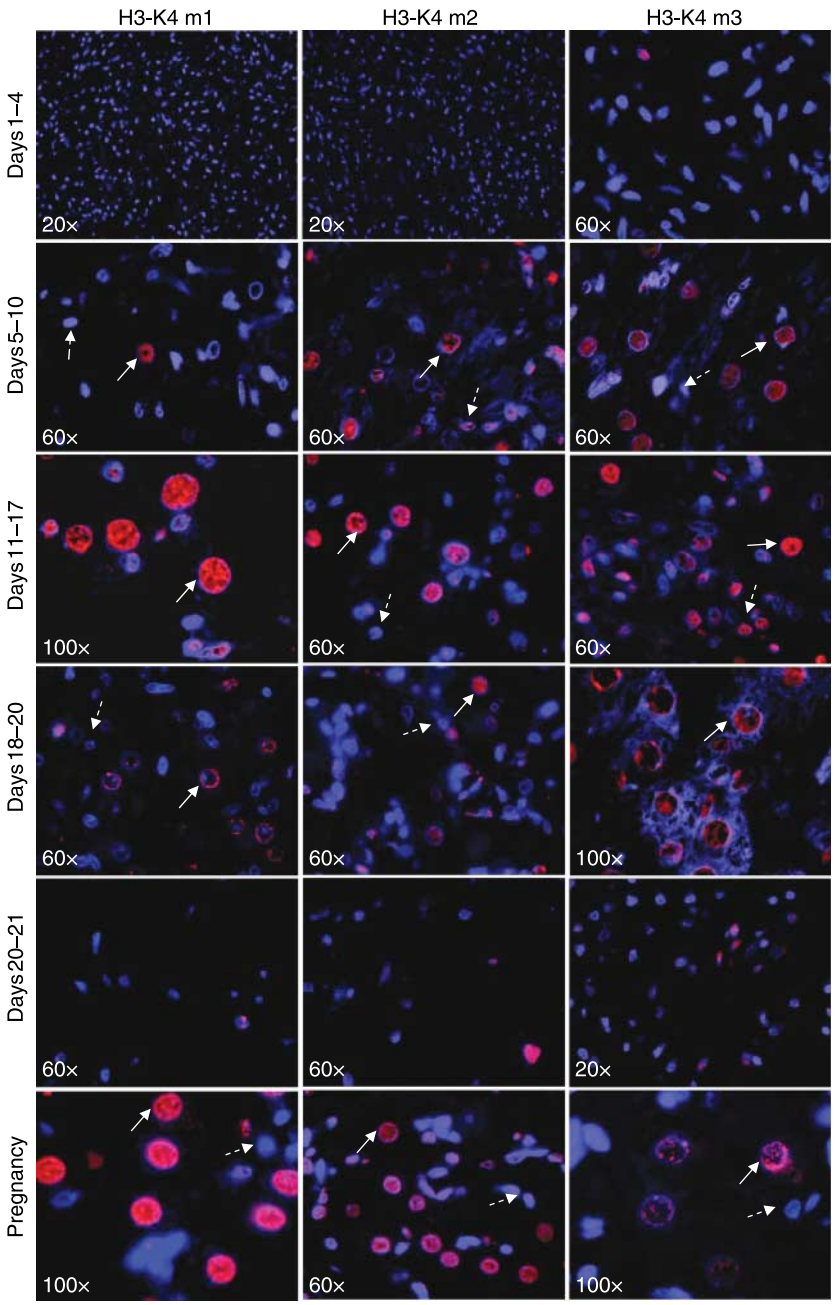

Figure 4 Immunofluorescent localization of methylation patterns of histone H3 at lysine 4 (H3-K4) during luteinization. Corpus luteum tissue determined to be from cycling pigs from days 1–4, 5–10, 11–17, 18–20, 20–21, or pregnancy was analyzed using immunofluorescence to determine the relative level and distribution of H3-K4 mono-methylation (H3-K4-me1, red), H3-K4 di-methylation (H3-K4-me2, red), and H3-K4 tri-methylation (H3-K4-me3, red). DAPI counter staining (blue). Magnification for each image is indicated. Solid arrows indicate large luteal cells and dashed arrows indicate small luteal cells.

was detected in granulosa cells from the primary to tertiary stage. A remarkable upregulation of H3-K4 methylation occurred in granulosa cells of the large antral follicle, with the strongest signals detected in the innermost antral granulosa and the cumulus. The appearance of H3-K4 methylation in antral follicles coincides with major functional and morphological changes as the preantral granulosa separate into populations, which form cumulus and the mural granulosa. Interestingly, H3-K4 methylation signals decreased in granulosa cells with distance from the oocyte. The cells displaying H3-K4 methylation in antral follicles have been shown to be a specialized granulosa population serving a unique role in the coordinate signaling between the oocyte and somatic cells (Eppig 2001). In antral follicles, the oocyte is thought to direct behavior of the granulosa in part by production of TGF-β signaling molecules, namely BMP15 and GDF9 (Eppig 2001). Co-expression of BMP15 and GDF9 acts on the granulosa to regulate FSH responsiveness and granulosa cell proliferation. The temporal appearance of H3-K4 methylation in the granulosa suggests a potential link to TGF-β signaling and FSH-dependent maturation to the

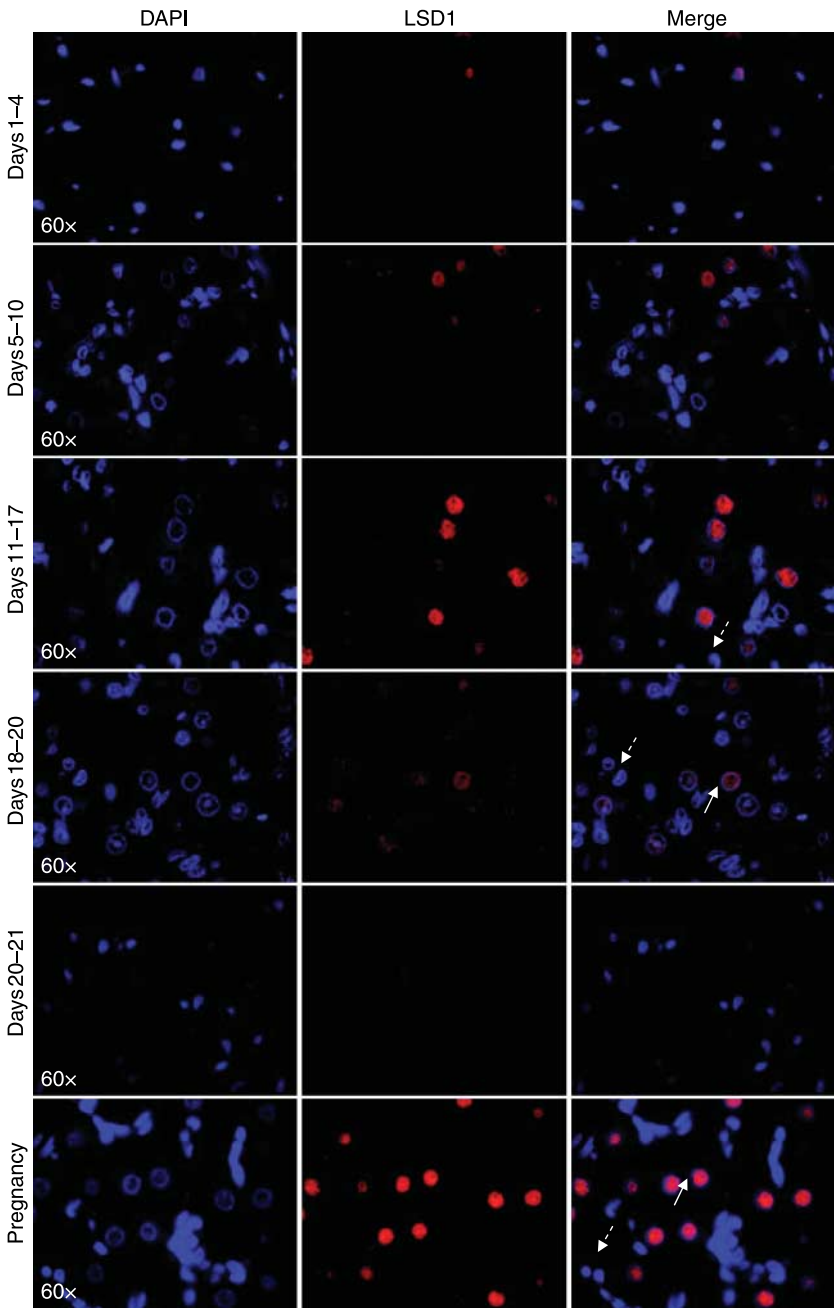

Figure 5 Immunofluorescent localization of lysine-specific demethylase 1 (LSD1/KDM1) during luteinization. Corpus luteum tissue determined to be from cycling pigs from days 1–4, 5–10, 11–17, 18–20, 20–21, or pregnancy was analyzed using immunofluorescence to determine the relative level and distribution of LSD1 (red). DAPI counter staining (blue). Magnification for each image is indicated. Solid arrows indicate large luteal cells and dashed arrows indicate small luteal cells.

preovulatory state. The upstream regulators and gene targets of H3-K4 methylation in mural and cumulus granulosa remain to be elucidated.

Ovulation is mediated by a series of morphological and gene-regulated events that eventually lead to the rupture of the follicle and release of the oocyte (Richards 2005). In order to gain insight into the regulatory pattern of H3-K4 methylation as ovulation approaches, we collected ovaries from synchronized gilts at 0, 24, and 38 h post-hCG injection and performed immunohistochemistry on dissected segments from the granulosa wall. This injection schedule mimics the LH surge and as a consequence, granulosa cells commence differentiation towards luteinization and transformation into the progesterone-secreting corpus luteum (Murphy *et al.* 2001). Although the eCG/hCG protocol has been widely used to promote follicular growth and synchronize ovulation in pigs, it is worth considering that this treatment may produce different outcomes compared with follicles collected from natural cycling gilts. At 0 h post injection, the levels of H3-K4 methylation were strongest in the mural granulosa, then significantly decreased by 24–38 h post injection when little H3-K4 methylation remained in the granulosa wall. Prostaglandin biosynthesis is a critical regulator of the ovulatory process and the prostaglandin

endoperoxide synthase COX2 is transiently expressed in the granulosa prior to ovulation (Sirois *et al*. 2004). It is worth considering that the expression of key ovulatory-associated genes, like *COX2*, could be under the control of H3-K4 methylation. While levels of H3-K4 methylation were low in granulosa at what would have been just prior to ovulation in the synchronized pigs, methylation in large luteal cells greatly increased as differentiation of corpus luteum progressed. These strong signals for H3-K4 methylation in large luteal cells could represent epigenetic control of genes required for progesterone secretion. As luteinization proceeds, in porcine large luteal cells, there is a dramatic upregulation of genes associated with sterol synthesis such as *STAR*, cholesterol side-chain cleavage enzyme (P450scc) and Niemann-Pick C1 (LaVoie *et al*. 1997, Pescador *et al*. 1999, Gevry *et al*. 2002). Similarly, in pregnancy, high levels of H3-K4 methylation were present in large luteal cells and a role for H3-K4 methylation in control of steroidogenic genes is suggested. Interestingly, LSD1 (KDM1) is strongly expressed in the mouse ovary in comparison with other somatic tissues (Godmann & Kimmins unpublished) and, in this study, it was found to occupy a highly restricted distribution being localized principally in large luteal cells, the supposed descendents of the granulosa cell population (Murphy *et al*. 2001). The co-localization of LSD1 (KDM1) and H3-K4 di- and tri-methylation in large luteal cells suggests that there is a functional interplay between H3-K4 methylation and LSD1 (KDM1). In terms of gene regulation, LSD1 (KDM1) may remove methylation at gene-specific targets associated with sterol synthesis and responsiveness to IGFs, prolactin, or gonadotropins. *In vitro* treatment of granulosa cells followed by chromatin immunoprecipitation will be informative in determining the gene-specific targets and regulation of LSD1 (KDM1) activity.

The oocyte nucleus or GV displays unique chromatin configurations that are altered during oocyte maturation. In the mouse, H3-K4 methylation has been implicated in the chromatin remodeling and epigenetic maturation associated with oocyte maturation (De La Fuente 2006). When oocytes display what has been termed an NSN they have been determined to have high rates of transcription, while in the SN state, there is global silencing (Bouniol-Baly *et al*. 1999, De La Fuente 2006). The high levels of H3-K4 methylation detected in non-surrounded pig oocytes indicate that, as in the mouse, these epigenetic modifications could play a significant role in regulation of oocyte transcription. Indeed, a critical role for histone H3 tri-methylation in the ovary has been established by gene knockout of histone methyltransferase Meisetz, where females are infertile (Hayashi *et al*. 2005).

Future investigation on the interplay between epigenetic events and endocrine and ovarian regulators will lead to a better understanding into the regulation of follicular development and allowing development of new techniques for assisted reproduction.

Materials and Methods

All chemicals were obtained from Sigma–Aldrich unless otherwise indicated.

Animals

All animal procedures were approved by the Animal Care and Use Committee of McGill University, Montreal, Canada. One ovary per gilt was collected at the slaughterhouse from ten prepubertal gilts immediately after evisceration. At collection, ovaries were examined to confirm prepubertal status by morphological evaluation for the absence of ovulated follicles, corpus hemorrhagicum, and corpora lutea. Ovaries were rinsed twice in cold PBS and 8×8× 8 mm fragments were cut and placed in a solution of 4% paraformaldehyde at 4 °C for 12 h with rocking. Ovarian fragments were then washed thrice in PBS at 4 °C and transferred to 70% EtOH. Tissues were processed for embedding in paraffin and sectioned using standard histological protocols. Immunofluorescent staining was performed on 3 μm thick sections. The corpora lutea samples were prepared from abattoir-derived ovaries from cyclic gilts at various stages of the estrous cycle and from pregnant sows using the same protocol. Ovaries were collected from gilts at the early, mid, late, and regressing stages of corpus luteum (CL) lifespan according to the morphological criteria described by Pescador *et al*. (1999). A total of three to five ovaries were analyzed for each CL stage.

Ovariectomy and isolation of follicles after synchronized ovulation

Prepubertal crossbreed (Landrace×Yorkshire) gilts with body weight of 90–95 kg and ∼145 days old were injected intramuscularly with 750 IU eCG (Folligon; Intervet, Whitby, ON, Canada) to induce follicular development. After 72 h, 500 IU hCG (Chorulon, Intervet) was given to induce ovulation. Four to five animals were randomly allocated to each of three groups for ovary collection at 0, 24, and 38 h following hCG treatment, considering the 42±2 h after hCG treatment for ovulation (Downey & Ainsworth 1980). Ovaries were collected by ovariectomy via mid-ventral laparotomy under general inhalation anesthesia (Coté 2001). Both ovaries were collected from each gilt and transferred to HEPES-buffered tissue culture medium (Invitrogen Life Technologies). Follicles were measured using a caliper and all follicles larger than 4 mm diameter were dissected from ovarian stroma. Follicular fluid was aspirated with a 23 gauge needle and the follicular wall was cut into three to four pieces. Tissues were processed for embedding in paraffinand sectioned using standard histological protocols.

Immunofluorescence

Briefly, tissues were deparaffinized with Citrisolve (Fisher Scientific Canada, Ottawa, Ontario) and rehydrated through three changes of alcohol. After washing in PBS-Brij (PBS with Brij 35 0.03%) for 10 min, antigen retrieval was performed by incubating tissue sections in sodium citrate buffer for 5 min in a pressure cooker. Sections were cooled to room temperature followed by washing thrice in PBS-Brij. The sections were subsequently blocked for 1 h in PBS-Brij with 10% of normal goat serum and 2.5% BSA then incubated with either polyclonal rabbit anti-H3-K4 mono-methyl (Abcam Inc., Cambridge, MA, USA; ab8895, 1/200), or anti-H3-K4 di-methyl (Abcam; ab7766, 1/200), or anti-H3-K4 tri-methyl (Abcam; ab8580, 1/200), or anti-LSD1 (KDM1; ab17721, 1/200), or control rabbit IgG (Jackson Immunoresearch Laboratories Inc., West Grove, PA, USA) at the same concentration as the primary antibody with rocking, overnight at 4 °C. Primary antibodies were detected using AlexaFluor 594-anti-rabbit and 488-anti-mouse (Molecular Probes Inc., Eugene, OR, USA) used at 1/1000. Cells were counterstained with DAPI. Immunofluorescent signals were measured using a Nikon eclipse 80i microscope and recorded by a Retiga 2000R monochromo digital camera (QImaging, Surrey, BC, Canada). The intensity level was evaluated on synchronized ovulation samples (0, 24, and 38 h after hCG) for mono-, di-, and tri-methylated H3-K4. All images were captured using the same settings and saved in TIFF format. The grayscale signal of 25 fields was analyzed for each replicate using the SimplePCI Imaging Software (Complix Inc., Sewickley, PA, USA).

Statistical analysis

Intensity level measurements were compared across the interval following the ovulatory stimulus (0, 24, and 38 h after hCG) for each marker. Data were analyzed by one-way ANOVA followed by Tukey–Kramer HSD test for multiple comparisons using the JMP software (SAS Institute Inc., Cary, NC, USA). Differences were considered to be statistically significant at the 95% confidence level ($P<0.05$).

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