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Epigenetic control of folliculogenesis and luteinization

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Abstract

Folliculogenesis and luteinization are characterized by irreversible and profound physiological and morphological transformation processes, which eventually culminate in the provision of fertilizable eggs and the conversion of the estrogen producing follicle into a progesterone producing corpus luteum. All these processes are preceded by complex alterations of the gene expression profiles in the somatic cell layers granulosa and theca. It has been well established that epigenetic mechanisms, such as DNA methylation, histone modification and local changes of the chromatin structure, are essentially involved in cell type-specific gene activation and silencing. This short review will mainly focus on epigenetic processes that are induced by the gonadotropins FSH and LH during late folliculogenesis and luteinization. Data will be presented demonstrating that histone modification and chromatin modulation, but also DNA methylation are involved in the changing gene expression profiles during folliculogenesis and luteinization. Hence, these epigenetic mechanisms have to be considered to understand the control of the female reproductive cycle and pregnancy as well as pathological aberrations.

Keywords: chromatin, DNA-methylation, estrogen, gene expression, histone, luteinizing hormone, progesterone.

Introduction

The provision of fertilizable eggs during ovulation is the final aim of folliculogenesis that is characterized by precisely controlled growth and differentiation processes. After ovulation the remaining somatic cell layers of the follicle, granulosa and theca, undergo an irreversible physiological and morphological reconstruction that eventually leads to the formation of a functional corpus luteum. Folliculogenesis that is characterized by recruitment, selection, dominance, and ovulation or atresia (Bao and Garverick, 1998) and luteinization are mainly under the control of the pituitary-derived gonadotropins follicle stimulating hormone (FSH) and luteinizing hormone (LH). Fully differentiated follicles and corpora lutea are important endocrine glands themselves, which produce large amounts of estrogens and progestins, respectively. Both are important regulators of the estrous cycle and pregnancy. Besides these steroid hormones, granulosa, theca and the oocyte itself produce a variety of other endocrine, paracrine and autocrine acting key factors as IGF-I, activin, inhibin and several factors of the TGF-beta superfamily (Knight and Glister, 2006) that are also important regulators of folliculogenesis, luteinization, estrous cycle and pregnancy. The major steroid hormone that is produced by dominant follicles is 17-beta-estradiol (E2). It plays an essential role during the female reproductive cycle acting via classical receptor mediated pathways and rapid, alternative pathways (Ivanova *et al*., 2002) on a variety of target tissues. In the bovine follicle, theca and granulosa are cellular layers that are separated by a basement membrane with the granulosa directly bordering the antral cavity. The "two-cell hypothesis" (Hillier *et al*., 1994) is based on the fact that the theca expresses all proteins and enzymes necessary for androgen synthesis including steroidogenic acute regulatory protein (STAR), the cytochrome P450 cholesterol side-chain cleavage enzyme (P450SSC), 3-beta-hydroxysteroid dehydrogenase (3β-HSD) and steroid 17-alpha-hydroxylase/17,20 lyase (P450C17). Aromatase cytochrome P450 (P450AROM) the key enzyme of estrogen biosynthesis, is only expressed in the granulosa, which otherwise does not contain considerable amounts of P450C17 (Bao *et al*., 1997). Hence, estrogen biosynthesis in the granulosa depends on androgen precursors produced by the neighboring theca. Granulosa and theca can be clearly distinguished by their responsiveness to the gonadotropins FSH and LH. Receptors for FSH are expressed almost exclusively by the granulosa virtually throughout all follicular stages. In contrast, receptors for LH are predominantly found in the theca of preantral as well as of tertiary follicles. Only large dominant follicles express LH receptors in their granulosa too (Bao and Garverick, 1998).

Ovulation and luteinization are initiated by the preovulatory LH surge. Two genes in particular, *CYP17* and *CYP19*, have been shown to be strongly down-regulated by LH in the bovine theca and granulosa, respectively (Voss and Fortune, 1993; Conley *et al*., 1995; Nimz *et al*., 2009). In a recent study in which the transcript abundance levels of several genes encoding key molecules of steroid biosynthesis and hormone receptors (FSHR, LHR, GHR) were analyzed, it was found that late preovulatory follicles show a transient gene expression profile that is clearly different from both, the preceding follicular and subsequent luteal stages (Nimz *et al*., 2009). For example, *CYP11A1* and *HSD3B*, which encode key enzymes of progesterone synthesis, are transiently downregulated after the

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preovulatory LH surge (Nimz *et al*., 2009) in granulosa and theca, but remarkably upregulated in fully luteinized cells after ovulation (Lenz *et al*., 2004; Vanselow *et al*., 2010).

The question arises which mechanisms control these well orchestrated changes of gene expression profiles during different stages of folliculogenesis and luteinization that are mainly triggered by gonadotropins. A complex combination of transcription factors and associated co-factors along hormone-activated signalling pathways obviously direct the activation and de-activation of target genes. However, increasing evidence has been accumulated during the last years that particularly the recruitment of enzymes capable of chromatin remodelling to a gene's regulatory regions are important prerequisites for transcription initiation or silencing (Berger, 2007) .

The present article was aimed to briefly review new data on those epigenetic mechanisms of gene regulation as DNA-methylation, histone modification and modulation of the chromatin structure that may be involved in the control of folliculogenesis, ovulation and luteinization. Generally, DNA methylation, histone modification and chromatin structure are considered intimately connected (Cheng and Blumenthal, 2010). However, histone modification and DNA methylation can have different roles in gene silencing, with histone modifications providing labile transcriptional repression and DNA methylation being a more stable silencing mark that is not easily reversed (Cedar and Bergman, 2009).

Histone modification

The N-terminal domain of histone proteins protrudes from the core domain and is subject to chemical modifications, such as acetylation, methylation, ubiquitination, or phosphorylation at specific residues (Nan *et al*., 1998; Ng and Bird, 1999; Lavrov and Kibanov, 2007; Kondo, 2009; Cheng and Blumenthal, 2010). Locus-specific histone modifications referred to as "histone code" are associated with the degree of chromatin condensation and thus with accessibility of the gene to transcription factors. Deciphering of this code may therefore allow to predict the transcription status of the respective chromatin region (Strahl and Allis, 2000; Jenuwein and Allis, 2001).

During the last years an increasing role of histone modification in controlling ovarian function has been established (Lavoie, 2005). In the porcine ovary, genome wide methylation of histone H3 at lysine 4 (H3-K4), a modification that is generally associated with gene activation, was found to change in a differentiation- and cell type-specific manner, thus suggestively serving an essential regulatory role during folliculogenesis (Seneda *et al*., 2008). A variety of factors and associated pathways have been identified that influence histone modification during folliculogenesis. Protein kinase A (PKA) seems to be an important nodal point that connects FSH action with chromatin remodeling and thus transcriptional facilitation (Hunzicker-Dunn and Maizels, 2006). It was found that histone H3 phosphorylation of serine 10 (H3-S10) is linked to mitosis and differentiation of rat granulosa, an effect that is induced by FSH and mediated by PKA action. This facilitation of PKA-dependent gene transcription eventually leads to the preovulatory phenotype in follicles of the rat (DeManno *et al*., 1999). Another study in rat granulosa demonstrated that FSH stimulates PKA-mediated histone H3 phosphorylation and acetylation leading to activation of serum glucocorticoid kinase, inhibin alpha and c-fos genes suggestively by selective reorganization of the corresponding promoters into a more accessible configuration (Salvador *et al*., 2001). In human granulosa an FSH induced estrogen receptor-(ER)-beta coactivator (GIOT-4) has been isolated, which recruits the SWI/ANF-type complex (ATP-dependent chromatin-remodelling complex) and thus induces histone modification via the histone acetyl transferase (HAT). This demonstrates a novel regulatory convergence between the gonadotropin signalling cascade and ER-beta mediated transcription in the ovary (Kouzu-Fujita *et al*., 2009). Particularly in the case of the *STAR* gene encoding the steroid acute regulatory protein, which is involved in cholesterol transportation into the mitochondria as the initial step of steroid biosynthesis, it has been demonstrated that gonadotropin-induced histone modification can be targeted to specific promoter regions. In porcine granulosa FSH increases H3-K9 and H3-K14 acetylation within the proximal region of the *STAR* promoter thus facilitating transcription. This effect was antagonized by EGF in this way reducing *STAR* mRNA abundance (Rusovici *et al*., 2005). In the mouse administration of hCG increased *STAR* transcription and initiated a rapid loss of the silencing H3-K9 dimethyl mark from the corresponding promoter in the granulosa (Hiroi *et al*., 2004). Epigenetic modification of the proximal *STAR* promoter was also observed in macaque and human granulosa, where enhanced transcription during luteinization was associated with acetylation of H3 (but not H4; Christenson *et al*., 2001). In cultured bovine granulosa, under luteinizing conditions expression of *STAR* significantly increased after 72 h. This was associated with histone H3 acetylation within the corresponding promoter region (Shimizu *et al*., 2009). Several other genes involved in steroidogenesis, have also been shown to be regulated by epigenetic mechanisms. Transcription of the *NPC-1* gene in porcine granulosa, encoding Niemann-Pick C-1, a protein that is involved in the network of intracellular cholesterol homeostasis, is modulated via H3

acetylation of its promoter (Gevry *et al*., 2008). In porcine granulosa it was shown that Krüppel-like (transcription-) factors are involved in the LH/IGF-1-induced repression of the low density lipoprotein receptor (*LDLR*) and *CYP11A1* genes by recruiting inhibitory complexes containing histone deacetylase (HDAC) corepressors to the corresponding promoter regions (Natesampillai *et al*., 2008). Interestingly, some undesirable side effects of VPA (valproate) treatment on ovarian function could be explained by the ability of VPA to act as a HDAC inhibitor (Nelson-DeGrave *et al*., 2004). Patients treated with VPA, a short-chained fatty acid with antimanic properties develop polycystic ovary syndrome-like symptoms including weight gain, hyperandrogenemia, and hyperinsulinemia. In these patients an increased androgen production caused by increased transcription of steroidogenic genes like *CYP17* and *CYP11A1* was found in cells of the theca. Eventually this dysregulation could be traced back to augmentation of transcription of these genes by histone H3 acetylation of corresponding promoters (Nelson-DeGrave *et al*., 2004).

DNA methylation

In vertebrates, methylation of cytosines to 5-methyl-cytosine, which occurs mostly in the context of CpG dinucleotides, imprints a specific methylation pattern on the DNA sequence and usually serves to properly silence genes in a tissue-specific manner during development (Ng and Bird, 1999). The majority of CpGs are methylated within the genome with the exception of those that are clustered within CpG-islands. Unclustered CpGs that are located proximal to active start sites of transcription tend towards lower methylation levels (Eckhardt *et al*., 2006). Generally, transcriptional silencing that is associated with DNA methylation plays a role for protection against intragenomic parasites (Walsh *et al*., 1998) and in carcinogenesis (Jones and Baylin, 2002). But also essential regulatory processes during mammalian development as genomic imprinting or X-chromosome inactivation (Li, 2002) are closely associated with DNA methylation of CpG islands. However, the potential role of DNA methylation in generating typical differentiation- and tissue-specific gene expression profiles or in the regulation of CpG-poor promoters is less well established. To date, it is also not clear whether DNA methylation changes during adult life in the course of differentiation processes such as folliculogenesis and luteinization.

In a previous study it has been shown that the abrupt shutdown of alpha S1-casein synthesis in mammary epithelial cells during acute mastitis is clearly connected with DNA de novo methylation around a STAT5-binding enhancer element in the corresponding *CSN1S1* gene (Vanselow *et al*., 2006). In the bovine and ovine placenta high-level expression of the *CYP19A1* gene clearly coincides with hypomethylation of the corresponding promoter (Fürbass *et al*., 2001, 2008; Vanselow *et al*., 2008). Gene- and cell type specific de novo DNA methylation, however, suggestively also occurs during estrous cycle in the bovine ovary. In case of the oxytocin locus it has been found that promoter methylation and chromatin compaction correlate with up- and down-regulation of gene expression in differentiating bovine granulosa cells (Kascheike *et al*., 1997). As mentioned above, expression levels of *CYP19A1* are very high in granulosa of large dominant bovine follicles (Lenz *et al*., 2004). In cells of the corpus luteum, however, only residual or even undetectable levels of *CYP19A1* transcripts were found (Vanselow *et al*., 2005). This coincides with the observation that CpG dinucleotides within the corresponding promoter are basically unmethylated in granulosa but methylated in luteal cells, suggesting that DNA methylation may be involved in silencing gene expression during luteinization. In a recent study, expression and methylation levels of the *CYP11A1*, *HSD3B* and *CYP19A1* genes were compared in theca and granulosa before and after the preovulatory LH surge (Vanselow *et al*., 2010). It was demonstrated that most CpGs located proximal to the respective start sites of transcription showed very different methylation levels in theca and granulosa, with very low levels in granulosa and significantly higher levels in theca. In contrast, liver samples as non-expressing negative controls showed highest methylation levels at these CpGs. Thus, all three genomic sequences meet the criteria of "tissue-specific differentially methylated regions" (T-DMRs; Eckhardt *et al*., 2006). The data also indicated a significant, non-linear connection between methylation of CpGs located proximal (i.e. closer than about 300bp) to the respective start site of transcription and gene expression levels: methylation levels above 25% seem to preclude high level gene expression, whereas low level methylation (<25%) seems to be an essential but not sufficient condition for high level gene expression. This is also perfectly in line with the observation that liver samples, which do not express *CYP11A1*, *HSD3B* or *CYP19A1*, show highest levels of methylation, particularly of proximal CpGs. Thus, the chromatin of the *CYP19A1* promoter might be condensed and repressed in theca (and also in liver) because of its high methylation levels in this cell type. Expression data demonstrating that *CYP19A1* transcripts are barely detectable in theca (Voss and Fortune, 1993; Nimz *et al*., 2009) are consistent with this idea. In granulosa, however, the same promoter is completely unmethylated independent of the differentiation status of the follicle, suggesting de-condensed, open chromatin. The same study, however, demonstrated in addition that methylation levels of individual CpGs were similar in follicles before and after the preovulatory LH surge. On one hand the fact that in the late preovulatory granulosa very low

methylation levels of *CYP19A1* coincide with very low expression levels (Nimz *et al*., 2009) demonstrate that apart from permissive DNA methylation levels, other essential factors and conditions for high level expression are no longer present in this tissue after the LH surge. On the other hand, these data also clearly indicate that DNA methylation is not involved in the profound, LH triggered preovulatory change of the gene expression profile, because the downregulation of gene expression is not reflected by the DNA methylation levels. In fully luteinized granulosa cells, however, methylation levels of proximal CpGs of *CYP19A1* were between 30% and almost 50%. Considering that the same CpGs were completely unmethylated in granulosa of large dominant follicles, this clearly indicates de-novo methylation. This is perfectly consistent with expression data: *CYP19A1* transcripts are highly abundant in pre-LH granulosa, but could barely be detected in luteal cells. Taken together, these data demonstrate that DNA methylation is not involved in the transient or permanent preovulatory down-regulation of gene expression. However, the data also strongly suggest that the proximal *CYP19A1* promoter is methylated during luteinization and that this methylation may be important for permanent silencing of this promoter in luteal cells. Thus, DNA methylation may be important for stabilizing the CL-specific gene expression profile.

Chromatin structure

Although DNA methylation, histone modification and chromatin modification are considered intimately connected (Cheng and Blumenthal, 2010), data on promoter-specific histone modification and DNA methylation generate only indirect information on the structure of the corresponding chromatin. In a recent study the degree of gene- and cell type-specific chromatin condensation around the transcription start sites of *HSD3B1*, *CYP17A1* and *CYP19A1* was determined by CHART-PCR using DNase I as an accessibility agent in granulosa and theca of bovine follicles before and after the preovulatory LH surge. The data clearly indicated that in the case of *CYP19A1,* the degree of chromatin condensation was remarkably different between theca and granulosa but also before and after the LH surge (unpublished data). Lowest condensation was found in the granulosa of E2 active follicles and the highest degrees of condensation was found in theca after LH. The chromatin condensation was similar in granulosa after LH and in theca before LH. This matches the gene-specific expression profile that is characterized by very high levels in granulosa, but not in theca of large dominant follicles and the almost complete downregulation after LH (Lenz *et al*., 2004; Vanselow *et al*., 2005; Nimz *et al*., 2009). The expression pattern of *CYP17A1*, however, was largely complementary with very high levels in theca, absence in granulosa and complete downregulation by LH (Conley *et al*., 1995; Bao and Garverick, 1998; Nimz *et al*., 2009). This expression pattern was mainly reflected by the chromatin condensation profile with high degrees of condensation in all granulosa samples and in theca after LH, and significantly lower condensation in theca before LH. The chromatin condensation of *HSD3B1* increased after LH in both theca and granulosa. This finding was in agreement with reduced, but not completely silenced expression of *HSD3B1* in these tissues. Taken together, these data clearly demonstrate that the preovulatory LH-induced chromatin condensation is not an unspecific, genome-wide effect, but rather gene- and tissue-specific.

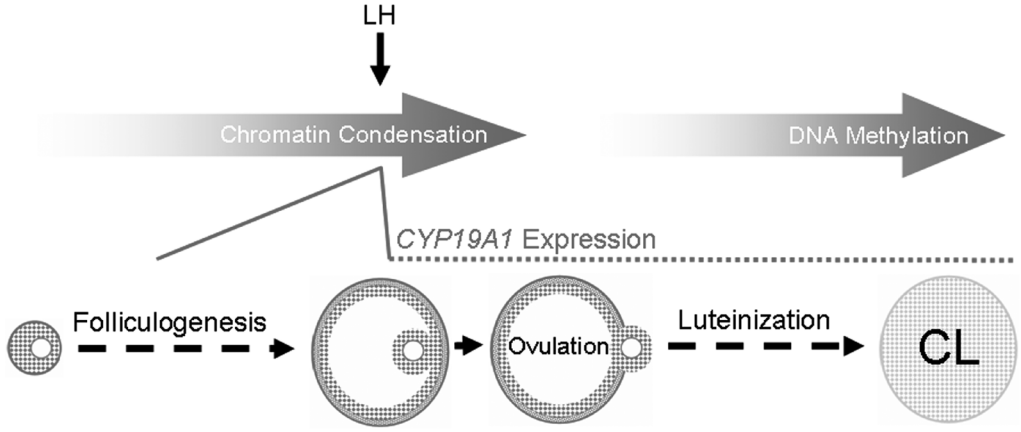

Figure 1. Chronological order of epigenetic events at the *CYP19A1* gene in granulosa and granulosa lutein cells during folliculogenesis and luteinization in the bovine. Expression of *CYP19A1* increases during follicular growth (gray line) and is rapidly down-regulated to almost undetectable levels (broken gray line) following the preovulatory LH surge. LH also initiates rapid condensation of *CYP19A1* chromatin and postponed de-novo methylation several days later in fully luteinized large (granulosa) lutein cells. Preantral, large dominant and ovulating follicles as well as the emerging corpus luteum (CL) are symbolized underneath. The time axis is not drawn to scale.

In summary, these data and the data on DNA methylation in bovine follicles (see above) strongly suggest that DNA methylation-independent chromatin condensation is involved in the LH-induced downregulation of *CYP19A1*, *CYP17A1* and *HSD3B1* expression. In the late preovulatory follicle, gene expression might be rapidly silenced by LH-induced chromatin compaction. The permanent fixation of the silent status, however, might happen several days later in fully luteinized granulosa lutein cells by promoter methylation (Vanselow *et al*., 2010). By reference to data on expression and epigenetic modulation of *CYP19A1* in granulosa during folliculogenesis and luteinization this chronological order of events is shown in Fig. 1.

Conclusion

Experimental evidence has been collected during the last years demonstrating that epigenetic mechanisms as histone modification and chromatin modulation, but also DNA methylation are involved in the changing gene expression profile during folliculogenesis and luteinization. Therefore, these mechanisms have to be considered to understand the molecular mechanisms that control the female reproductive cycle and to elucidate pathological aberrations.

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