Molecular mechanisms of luteinization

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Dynamic changes in steroidogenesis occur in ovarian granulosa cells during ovulation after the LH surge. The ovulatory LH surge induces rapid up-regulation of steroidogenic acute regulatory (StAR) protein and rapid down-regulation of aromatase (Cyp19a1) in granulosa cells undergoing luteinization during ovulation. These rapid changes in StAR and Cyp19a1 gene expression after the LH surge efficiently facilitate progesterone production, which plays a crucial role in ovulation and the following luteinization. Recently, it has become clear that epigenetic regulation such as histone modifications and DNA methylation play a key role in gene expression through the chromatin remodeling of the promoter region. This study reports the in vivo evidence that epigenetic mechanisms including histone modifications, DNA methylation and chromatin remodeling are involved in the rapid changes of StAR and Cyp19a1 gene expression in granulosa cells undergoing luteinization during ovulation.

Keywords: Cyp19a1; Epigenetics; Histone modification; Luteinization; StAR

Introduction

Dynamic changes in steroidogenesis occur in ovarian granulosa cells undergoing luteinization during ovulation after the LH surge. The ovulatory LH surge interacts with LH receptor and stimulates c-AMP mediated signal transduction pathway including epidermal growth factor-like factors (amphiregulin and epiregulin) and subsequent ERK-1 and -2 signals, and thereafter induces rapid up-regulation of steroidogenic acute regulatory (StAR) protein, a rate-limiting enzyme for progesterone synthesis [1], while the expression of aromatase (Cyp19a1), a key enzyme for estrogen synthesis, is rapidly suppressed after the LH surge [2]. These rapid changes in StAR and Cyp19a1 gene expression after the LH surge efficiently facilitate progesterone production, which plays a crucial role in ovulation and the following luteinization. The biological effect of progesterone via progesterone receptors is necessary for ovulation. Suppression of progesterone synthesis by 3b-hydroxysteroid-dehydrogenase inhibitors blocks ovulation in rats [3]. Progesterone receptor-null mice fail to ovulate due to the impaired follicle rupture [4]. However, it is unclear about molecular mechanisms for the rapid changes in StAR and Cyp19a1 gene expression in granulosa cells undergoing luteinization after the ovulatory LH surge.

The regulation of gene expression by transcription factors has been well accepted. Recently, it has become clear that epigenetic mechanisms such as histone modifications and DNA methylation play a key role in transcription through the chromatin remodeling of the promoter region. We have reported that histone modifications are involved in the regulation of gene expression of the human endometrium [5-8], and DNA methylation is involved in the pathogenesis of certain reproductive diseases such as endometriosis and uterine leiomyomas [9-14]. This study reports the in vivo evidence that epigenetic mechanisms including histone modifications, DNA methylation and chromatin remodeling are involved in the rapid changes of StAR and Cyp19a1 gene expression in granulosa cells undergoing luteinization during ovulation [15].
Gene expressions of StAR and Cyp19a1

The present study was reviewed and approved by the Committee for the Ethics on Animal Experiment in Yamaguchi University Graduate School of Medicine. Female Sprague-Dawley rats (21–24 days old) were injected subcutaneously with 15 IU of equine chorionic gonadotropin (eCG) followed by 15 IU of human chorionic gonadotropin (hCG) 48 hours after eCG injection to induce ovulation and luteinization. Granulosa cells were isolated before hCG (0 hours), and 4, 8, and 12 hours after hCG injection. StAR mRNA levels rapidly increased after hCG injection, reached a peak at 4 hours, and remained high until 12 hours after hCG injection (Fig. 1). In contrast, Cyp19a1 mRNA levels gradually decreased after hCG injection and were significantly lower at 8 and 12 hours compared with 0 hours (Fig. 1).

Regulation of gene expression by epigenetic mechanisms

In addition to transcription factors, epigenetic mechanisms such as DNA methylation and histone modifications are involved in transcriptional regulation. DNA methylation occurs at cytosines within CpG dinucleotides, which can silence gene expression by altering chromatin structure and preventing the binding of transcription factors [16,17]. Histone modifications also affect chromatin structure. Chromatin structure of the promoter is critical for the interaction of transcription factors with response elements [18,19]. Histone modifications, such as acetylation of histone H3 and histone H4 or trimethylation of the site of lysine 4 on histone H3 (H3K4me3) activate transcription by loosening the chromatin structure and allowing the recruitment of transcription factors to their response elements. On the other hand, histone modifications such as trimethylation of the site of lysine 9 and 27 on histone H3 (H3K9me3 and H3K27me3) inactivate transcription by causing the chromatin to become more condensed [19].

DNA methylation status of the StAR and Cyp19a1 promoters

The DNA methylation status at the CpG sites in the StAR promoter region (-1,722 to -13 bp) was analyzed by sodium bisulfite genomic sequencing (Fig. 2A). The promoter region until -500 bp is especially important for transcription because a number of binding sites of various transcription factors are located in this region. There are 6 CpG sites in the proximal promoter region. Before hCG injection (0 hours), the CpG sites in the proximal region were unmethylated, whereas the CpG sites in the distal region were methylated (Fig. 2A). These methylation statuses did not change at any time observed after hCG injection, suggesting that the

![Fig. 1. Changes in mRNA expression of StAR (A) and Cyp19a1 (B) in granulosa cells undergoing luteinization during ovulation. Granulosa cells were obtained from rats treated with equine chorionic gonadotropin before (0 hours) and 4, 8, and 12 hours after human chorionic gonadotropin injection. mRNA levels were measured by real-time reverse transcription polymerase chain reaction. Data were expressed as a ratio of StAR/Cyp19a1 mRNA to GAPDH (internal control). Each bar represents the mean ± SEM of five animals. The data were reported in the reference no. 15.](image-url)
Norihiro Sugino. Molecular mechanisms of luteinization

The proximal region of the StAR promoter between -550 bp and -13 bp is an important region for transcription.

The Cyp19a1 promoter II is located upstream of exon II, and is recognized as a tissue-specific promoter region in the rat ovary [20,21]. The region until -150 bp is critical for transcription. There are 2 CG sites in the promoter between -248 and -68 bp. Both CpG sites were unmethylated before (0 hours) hCG injection (Fig. 2B) and did not change after hCG injection, suggesting that this region is important for transcription.

**Histone modifications of the StAR and Cyp19a1 promoters**

The change in histone modifications of the StAR promoter region after hCG injection was analyzed by chromatin im-
munoprecipitation (ChIP) assay in the proximal region (proximal region 1, -257 to -42 bp; proximal region 2, -492 to -288 bp) and the distal region (-1383 to -1195 bp) (Fig. 3). The 5′-upstream region between -257 and -42 bp of the StAR gene (proximal region 1) is especially critical for the regulation of StAR gene expression. Since the change in histone modifications in the proximal region 2 was similar to those in the proximal region 1, the result of the proximal region 1 was only shown in Fig. 4. Active marks of histone modifications such as histone-H4 acetylation and H3K4me3 increased after hCG injection, whereas repressive marks such as H3K9me3 and H3K27me3 decreased after hCG injection (Fig. 4). Histone-H3 acetylation did not change after hCG injection (data not shown). On the other hand, in the distal region, active histone modification marks did not change after hCG (data not shown).

The change in histone modifications of the Cyp19α1 promoter II after hCG injection was analyzed by ChIP assay in the region between -169 and -15 bp (Fig. 3). Active marks of histone modifications such as histone-H4 acetylation and H3K4me3 decreased after hCG injection, whereas repressive marks such as H3K27me3 increased after hCG injection (Fig. 4). Histone-H3 acetylation also decreased after hCG injection in a manner similar to histone-H4 acetylation (data not shown).

It is unclear how the promoter region-specific histone modifications are regulated. Further studies on the recruitment of histone modification enzymes such as histone acetyltransferases, histone deacetylases, histone methyltransferases, or histone demethylases to each promoter region are needed.

### Chromatin structure in the StAR and Cyp19α1 promoters

To investigate whether the change in histone modifications in the StAR and Cyp19α1 promoter regions actually alters the chromatin structure of the promoters, DNase I accessibility assay was performed. In the proximal region 1 of the StAR, DNase I accessibility levels decreased after hCG injection, suggesting that the chromatin structure of the StAR promoter loosens after hCG injection (Fig. 5A). The change in chromatin structure of the proximal region 2 was similar to that in the proximal region 1. On the other hand, DNase accessibility levels in the Cyp19α1 promoter increased after hCG injection (data not shown). The change in histone modifications of the Cyp19α1 promoter II after hCG injection was analyzed by ChIP assay in the region between -169 and -15 bp (Fig. 3). Active marks of histone modifications such as histone-H4 acetylation and H3K4me3 decreased after hCG injection, whereas repressive marks such as H3K27me3 increased after hCG injection (Fig. 4). Histone-H3 acetylation also decreased after hCG injection in a manner similar to histone-H4 acetylation (data not shown).

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### Binding activities of C/EBP β and phosphorylated CREB to the StAR and Cyp19α1 promoters

Recent studies using knockout mice or microarray analyses have shown that CAATT/enhancer-binding protein (C/EBP)
Fig. 4. Histone modifications of the promoters of \textit{StAR} and \textit{Cyp19a1} in granulosa cells undergoing luteinization during ovulation. Granulosa cells were obtained from rats treated with equine chorionic gonadotropin before (0 hours) and 4 hours, and 12 hours after human chorionic gonadotropin injection. For histone modifications, histone-H4 acetylation (AcH4), trimethyl histone H3K4 (H3K4me3), trimethyl histone H3K9 (H3K9me3) and trimethyl histone H3K27 (H3K27me3) were analyzed by chromatin immunoprecipitation assay in each region of the \textit{StAR} promoter (proximal region 1, proximal region 2, and distal region) and the \textit{Cyp19a1} promoter (Fig. 3). The data of the proximal region-1 of the \textit{StAR} promoter are shown. AcH4 and H3K4me3 activate transcription while H3K9me3 and H3K27me3 inactivate transcription. Data were expressed as a ratio of the value of 0 hours. Values are mean±SEM of more than three independent experiments. *P<0.05 versus 0 hours. The data were reported in the reference no. 15.
β is a crucial transcription factor that induces StAR gene expression in granulosa cells undergoing luteinization [22,23]. On the other hand, cAMP-response element binding protein (CREB) up-regulates Cyp19a1 gene expression in granulosa cells under FSH stimulation [24-26]. We investigated whether these transcription factors actually bind to or detach from the
promoter region of StAR and Cyp19a1 after hCG injection. Binding activities of C/EBP β to the StAR proximal promoter region (proximal region 1) and of phosphorylated CREB to the Cyp19a1 promoter were analyzed by ChIP assay. Binding activities of C/EBP β to the StAR proximal promoter region increased after hCG injection (Fig. 5C). In contrast, the binding activities of phosphorylated CREB to the Cyp19a1 promoter region decreased after hCG injection (Fig. 5D), suggesting the detachment of phosphorylated CREB from the Cyp19a1 promoter after hCG stimulation because the protein level of phosphorylated CREB increased after hCG injection (data not shown).

Regulation of StAR gene expression by epigenetic mechanisms

The DNA methylation status of the StAR proximal promoter region was unmethylated. Furthermore, hCG stimulation caused modifications of histones in the StAR proximal promoter region and loosened the chromatin structure, allowing the transcription factor, C/EBP b, to access its response element of the StAR proximal promoter. This mechanism contributes to the rapid induction of StAR gene expression in granulosa cells undergoing luteinization after ovulatory LH surge.

Christenson et al. [27] reported that the level of histone H3 acetylation of the StAR proximal promoter was increased in luteinized granulosa cells obtained after the induction of ovulation compared with the level in non-luteinized granulosa cells in the macaque. Hiroi et al. [28] also reported that the H3K9 methylation level of the StAR proximal promoter was immediately decreased in luteinizing granulosa cells after hCG stimulation. Our results are consistent with these reports.

The changes in chromatin structure and histone modifications in the StAR promoter were more apparent in the proximal promoter region than the distal promoter region. This finding is consistent with a recent report that histone acetylation levels are high at the region surrounding the transcription start site and are gradually less acetylated at increasing distances from the transcription start site [29]. Chromatin remodeling in the StAR promoter is spatiotemporally regulated by the different types of histone modifications in the granulosa cells undergoing luteinization after hCG stimulation.

Regulation of Cyp19a1 gene expression by epigenetic mechanisms

Ovulatory stimulation also caused changes in histone modifications and chromatin structure in the Cyp19a1 promoter. hCG stimulation decreased levels of histone acetylation and H3K4me3 and increased levels of H3k27me3 in the Cyp19a1 promoter region, which inactivate transcription. Thus, hCG stimulation made the chromatin structure of the Cyp19a1 promoter region condensed through the alteration of histone modifications, allowing the transcription factor, phosphorylated CREB, to detach from its response element of the Cyp19a1 promoter. The change in histone modifications and chromatin structure of the Cyp19a1 promoter are closely associated with the rapid suppression of Cyp19a1 gene expression in granulosa cells undergoing luteinization by ovulatory stimulation.

The signaling pathway of cAMP-protein kinase A-CREB is a key signal for Cyp19a1 gene expression in granulosa cells under FSH stimulation [24-26]. This pathway is also induced by the LH surge. However, it has been unclear about the mechanism by which Cyp19a1 gene expression is suppressed in granulosa cells undergoing luteinization after the LH surge. This study showed the possible mechanism for the rapid suppression of Cyp19a1 gene expression in granulosa cells after ovulatory LH surge. The result that the binding activity of phosphorylated CREB to the Cyp19a1 promoter was decreased after hCG stimulation despite the constant protein expression of phosphorylated CREB in the nucleus strongly suggests the detachment of phosphorylated CREB from the Cyp19a1 promoter.

Conclusion

The present study showed that epigenetic changes (histone modifications and chromatin remodeling) occurred in the promoter regions of StAR and Cyp19a1 in granulosa cells undergoing luteinization during ovulation. Our results suggest that histone modifications and chromatin remodeling of the promoters are closely associated with the rapid changes of the expression of StAR and Cyp19a1 in luteinizing granulosa cells during ovulation, and provide a molecular mechanism for
the shift from estrogen synthesis to progesterone synthesis in granulosa cells undergoing luteinization after the LH surge.

**Conflict of interest**

No potential conflict of interest relevant to this article was reported.

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