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Tissue-specific promoter methylation and histone modification regulate *CYP19* gene expression during folliculogenesis and luteinization in buffalo ovary

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ABSTRACT

Aromatase, the key enzyme of estrogen biosynthesis, is encoded by the *CYP19* gene. The expression of *CYP19* gene is regulated in species- and tissue-specific manner by alternate use of different promoters. We have previously, cloned and characterized the tissue-specific promoter and tissue-specific transcripts in preovulatory (granulosa cells) and postovulatory (corpus luteum) structure of buffalo ovary. The present study was aimed to understand if epigenetic gene regulation through DNA methylation and histone modifications is involved in tissue-specific *CYP19* gene regulation during folliculogenesis and luteinization in buffalo ovary. Methylation analysis of five CpG dinucleotides of ovary specific proximal promoter II showed hypo-methylation in large follicle while hyper-methylation in corpus luteum. However, PI.1, the exclusive promoter responsible for residual *CYP19* gene expression in corpus luteum, was found to be hypermethylated. Analysis of histone modifications using ChIP assay revealed that the distal promoter (PI.1) of *CYP19* gene is ~40-fold more enriched with acetylated Histone H3 in corpus luteum than in the large follicle. This indicates that PI.1 chromatin was more accessible for transcription in corpus luteum as compared to large follicles. The chromatin accessibility for the proximal promoter (PII) in the preovulatory stage tends to be higher than the luteal tissue. However, the difference was not found to be significant. *In vitro* experiments showed the similar results. In conclusion, results of the present study suggests that tissue-specific methylation status of PII and chromatin remodeling through histone modifications of PI.1, coincides with the changes in expression of *CYP19* gene and thus are the regulatory mechanism controlling its tissue-specific expression and promoter activity during folliculogenesis and luteinization.

1. Introduction

A key endocrine marker of differentiating ovarian granulosa cells is the ability to synthesize estrogens. *CYP19* gene, encoding the enzyme aromatase cytochrome P450, catalyzes the final rate limiting step in the biosynthesis of estrogens from androgens [1]. It is one of the principal steroid hormones produced by the granulosa cells before their terminal differentiation into LH (luteinizing hormone) secreting corpus luteum [45]. It has been reported that the increased expression of aromatase mRNA from small to large follicles indicates the high 17β-estradiol synthesis in antral follicles, an essential requirement for the follicular development and maturation in bovine [3] and buffalo ovary [44]. Thus *CYP19* gene, catalyzing the biosynthesis of estrogens, is one of the crucial genes among the cluster of genes regulating the recruitment and selection of dominant follicle prior to ovulation.

The expression of *CYP19* mRNA is stimulated by follicle-stimulating hormone (FSH) in rats, humans and ruminants [35,46,17]. Both FSH [18] and estradiol [25] are critical regulators of folliculogenesis. Thus, the growing follicle produces an increasing amount of 17β-estradiol under the influence of FSH and growth factors. The transformation of estradiol secreting dominant follicle stage to the progesterone producing postovulatory structure (corpus luteum) is accompanied by profound molecular and morphological changes. This transition is associated with unique changes in the expression of many genes including *CYP19*, whose expression decreases after luteinization in ruminants [34]. It has been speculated that a switching mechanism may be involved in the ovulation/luteinization process [36].

In most mammals, expression of *CYP19* gene is regulated in tissue-specific manner by the alternative use of different promoters. Aromatase promoter-switching phenomenon was reported [8] in granulosa cells of cattle during follicular luteinization [4]. During growth of small to large ovarian follicles in cattle, *CYP19* transcripts were observed to be derived predominantly from promoter II [47]. However, these were almost exclusively derived from promoter I.1 in corpus luteum [26]. In bovine placenta, major

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transcript variant was from exon I.1 [20]. The ovary-specific transcripts are conserved among mammalian species whereas in cow and sheep placenta, different transcripts are expressed [19], suggesting that unrelated placenta-specific promoters are used in different species. In our previous study, we have reported that aromatase promoter-switching phenomenon in buffalo granulosa cells during follicular luteinization is characterized by a down regulation of promoter II- and an up-regulation of promoter I.1-derived transcript after differentiation of granulosa cells [11]. The molecular mechanism involved in this promoter switching regulating tissue-specific *CYP19* gene expression and thus its complex transcriptional regulation in buffalo ovary still remains to be elucidated.

In recent years, epigenetic mechanisms as DNA methylation and histone modifications have been implicated in complex regulation of *CYP19* gene [24,23]. These mechanisms exert effects that are essential to the regulation of gene expression in eukaryotes [48]. Epigenetic mechanisms including DNA methylation and chromatin modulation through histone modifications are involved in the changing gene expression profile during folliculogenesis and luteinization [21]. In vertebrates, methylation of cytosines that correspond to CpG dinucleotides has been found to be an important factor in the local organization of the chromatin structure and thus the long-term regulation of gene expression via epigenetic mechanisms. Generally, DNA methylation leads to transcriptional repression due to condensed chromatin structure [2,12,16]. Although, the tissue-specific pattern of DNA methylation is seen to be mainly established only during embryogenesis, its modulation also sometimes occurs during adult life [16]. DNA methylation levels not only show tissue – but also differentiation-specific differences [40]. Recently, we found that DNA methylation is also involved in stage specific *CYP19* gene expression in buffalo placenta [43]. Vanselow et al. [22] have shown that *CYP19* gene promoter region was almost unmethylated in the granulosa cells while methylated in corpus luteum. However, the expression of PI.1 derived transcripts in corpus luteum was not found sensitive to DNA methylation. Recently, inhibition of DNA methylation with 5-aza-2-deoxycytidine has been found to induce *CYP19* mRNA expression in breast adipose fibroblasts (BAFs) and breast cell lines [24]. In addition to DNA methylation, covalent modifications of histones and remodeling of chromatin structure have emerged as critical regulators of gene transcription [2,33]. The highly basic N-terminal histone tails projecting away from the core histone complex have a principal role in higher order chromatin structure and in interactions of histones with other chromatin-associated regulatory proteins [27]. For the initiation of transcription, specific DNA binding proteins recruit general transcription cofactors that are known to acetylate nucleosomal histones associated with the gene promoter resulting into the opening of chromatin structure [14]. This in turn attracts and activates the general transcription initiation complex to the specific promoter, thereby initiating transcription. Stocco [7] has shown that chromatin modifications are important for the cell-specific expression of aromatase in the mouse ovary. During trophoblast differentiation, estrogen/ER alpha exerts a positive feedback role, which promotes permissive histone modifications (acetylated Histone H3 (K9/14)) that are associated with induction of human *CYP19* gene transcription [38]. Acetylation of Histone H3 on lysine 14 is known to result in the reorganization of the promoters of FSH responsive genes into more accessible configuration for gene activation [28]. Hirori et al. [14] have shown that a combined code of transcription factors including reciprocal changes in histone modifications associated with active transcription and gene silencing control steroidogenic acute regulatory protein (STAR) gene expression in mouse MA-10 leydig cell tumor cell line. In view of the above, the likely possibility is that epigenetic modifications (DNA methylation and histone modifications) could be involved in the tissue-specific promoter switching mechanism of *CYP19* gene regulation. The objective of the present study was thus to understand if epigenetic mechanisms are responsible for hormonally regulated tissue-specific transcript expression of *CYP19* gene during folliculogenesis and luteinization in buffalo ovary.

2. Material and Methods

2.1. Collection of buffalo ovaries

Buffalo ovaries were collected from commercial abattoir, Delhi, within 10–20 min after slaughtering, in chilled normal saline (0.9% NaCl) containing penicillin (100 U/ml) and streptomycin (100 μg/ml) and transported to laboratory rapidly (approx within 4 h). All the tissues were washed at least five times in saline, disinfected once in 70% ethanol for 30 s and then washed again with saline and processed immediately.

2.2. Isolation of granulosa cells

Healthy, developing follicles were assessed by the presence of vascularized theca externa and clear amber follicular fluid with no debris. For *in vivo* studies, follicular fluid was aspirated from small ($\leqslant$5 mm), and large antral follicles ($\geqslant$8 mm), using 18 gauze needle and sterile, non-toxic, non-pyrogenic monoinjected brand syringes (Dispovan, 2.0 ml). The follicular fluid was collected in 15 ml centrifuge tube under sterile conditions while continuously maintaining the cells on ice. The granulosa cells were isolated by centrifugation at low speed (1500 rpm) to pellet out the cells. Cell pellets were used for total RNA and DNA isolation. The post-ovulatory tissue i.e. corpus luteum was excised with the help of sterilized scissors and kept in chilled saline till further processing. The appropriate amount (100 mg) of tissue required for DNA and RNA isolation was chopped into pieces and homogenized in DNAzol and TRIzol (MRC) respectively. For *in vitro* experiments (cell culture), follicular fluid was aspirated from small and medium antral follicles ($\leqslant$8 mm) using 18 gauze needle and sterile, non-toxic, non-pyrogenic monoinjected brand syringes (Dispovan, 2.0 ml). The fluid was collected in phosphate buffered saline (PBS) containing penicillin (100 U/ml), streptomycin (100 μg/ml) and amphoterecin B (1.25 μg/ml) in 15 ml centrifuge tube under sterile conditions while continuously maintaining the cells on ice. The granulosa cells were finally separated by centrifugation at low speed (1500 rpm) for 4–6 min to pellet out the cells. Cell number and viability were estimated in hemocytometer using trypan blue exclusion method.

2.3. RNA and DNA isolation

Total RNA and DNA were isolated from granulosa cells and tissues using TRIzol and DNAzol reagent (MRC) respectively following manufacturer's instructions. The RNA and DNA were quantified spectrophotometrically, DNA and RNA integrity was evaluated by normal and denaturing agarose gel electrophoresis, respectively.

2.4. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Complimentary DNA was synthesized from 1 μg of total RNA using random hexamers and the First strand cDNA synthesis kit (Fermentas, Germany) following the manufacturers instructions. The reaction mixture contained 1 μg of total RNA, 1 μl of random hexamer (0.2 μg/μl) and sH_2O to 11 μl. The contents were incubated at 65 °C for 10 min followed by 2 min incubation at room temperature. The reagents added further were: 4 μl of 5× reaction buffer (250 mM Tris–HCl, pH 8.3; 250 mM KCl, 20 mM $MgCl_2$, 50 mM