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Involvement of transcription factor GATA-4 in regulation of *CYP19* gene during folliculogenesis and luteinization in buffalo ovary

Rachna Monga [a], Sandeep Ghai [a], Tirtha Kumar Datta [b], Dheer Singh [a,*]

[a] *Molecular Endocrinology Laboratory, Animal Biochemistry Division, National Dairy Research Institute (NDRI), Karnal 132001, Haryana, India*
[b] *Animal Biotechnology Centre, National Dairy Research Institute (NDRI), Karnal 132001, Haryana, India*

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ABSTRACT

CYP19 gene encode aromatase, the key enzyme of estrogen biosynthesis, is regulated in species- and tissue-specific manner by alternate use of different promoters. Previously, we have reported the cloning and characterization of tissue-specific promoter and transcripts in buffalo ovary and placenta. In human and rat ovary, FSH induces the phosphorylation of transcription factor CREB (cAMP response element binding protein) through PKA pathway which binds to cAMP response element like sequence (CLS) in *CYP19* gene ovarian promoter. However, in buffalo as well as in bovine, *in silico* analysis of ovary specific promoter sequence identified a single base pair deletion in CLS site and is designated as CLS-like sequence. To understand if CLS with a point mutation is still a functional *cis*-element and is involved in FSH stimulated transactivation of *CYP19* gene in buffalo ovary, the present study was thus aimed to functionally characterize the role of buffalo CLS in *CYP19* gene transactivation. We also studied the involvement of GATA-4, having consensus binding sites in *CYP19* gene ovarian promoter in the vicinity of CLS during different stages of the buffalo estrus cycle. Reporter construct analyses and EMSA results showed that CLS is playing no significant role in *CYP19* gene regulation in buffalo ovary. Real time absolute quantification of GATA-4 showed the differential expression of GATA-4 mRNA during folliculogenesis and luteinization with significantly higher transcript abundance in large follicle in comparison to other tissues. Western blot analysis of granulosa cells nuclear protein isolated from different stage of follicular development (small and large follicles) and differentiation (corpus luteum) showed that abundance of phosphorylated GATA-4 (Ser261) was significantly higher in granulosa cell nuclear protein of large follicles as compared to small follicles and corpora lutea. Interestingly, binding studies using ChIP showed significantly enhanced binding to the *CYP19* gene promoter in large follicle which was seen to be declined in the luteal tissue. Similar results were obtained in the *in vitro* experiments as well. Finally, RNAi experiments were performed to validate the involvement of GATA-4 in *CYP19* gene regulation. Results of RNAi showed that knockdown of GATA-4 mRNA significantly declined *CYP19* gene mRNA as well as 17β-estradiol contents. In conclusion, result of the present study indicated that that in the absence of consensus CRE (cAMP response element); GATA-4 could be a downstream effector of cAMP/PKA pathway in regulation of *CYP19* gene during folliculogenesis and luteinization.

1. Introduction

A key endocrine marker of 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 has been reported that in the FSH responsive follicle, 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 [2] and buffalo ovary [3]. However, the LH surge transiently decreases FSH receptor and aromatase expression and activates genes needed for the dramatic increase in progesterone production associated with lutenization [4]. Thus, regulation of *CYP19* gene transcription is a critical step in terminal stage of follicular maturation.

In most mammals, expression of *CYP19* gene is regulated in tissue-specific manner by alternative use of different promoters [5,6]. Recently, we have cloned and characterized tissue-specific promoters and analyzed their epigenetic regulation [3,7–9]. However, the molecular mechanism involving the key *cis*- and *trans*-molecules regulating the tissue-specific *CYP19* gene expression and thus its complex transcriptional regulation in buffalo ovary still remains to be elucidated.

In general, in rats, humans and ruminants, the expression of *CYP19* mRNA is primarily stimulated by follicle-stimulating hormone (FSH) [10–12]. Detailed studies on its regulation in humans

* Corresponding author. Tel.: +91 184 2259135; fax: +91 184 2250042.
E-mail address: drdheer.singh@gmail.com (D. Singh).

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and rat showed that binding of FSH to its G-coupled receptors on granulosa cells results in an increase of intracellular cAMP levels, which, in turn phosphorylates certain transcription factors which bind to the functional promoter elements and thereby stimulates the expression of genes encoding steroidogenic enzymes [13]. Recent studies in bovine [14] and buffalo [15] granulosa cells have shown that IGF1 synergizes the effects of FSH in enhancing the expression of genes encoding steroidogenic enzymes and hormone production. IGF1 stimulated both PI3 Kinase and MAPK pathway by phosphorylating Akt and MAPK in completely serum free bovine granulosa cell culture [14]. FSH alone had no effect on this signaling molecule but produced synergistic response in Akt phosphorylation in the presence of IGF1. Therefore, similar molecules were recruited by FSH and IGF1 functioning through same signaling pathway resulting into enhanced gene expression and hormone production. In gonads, there are several cAMP-regulated genes which include aromatase (*CYP19*), steroid 17α-hydroxylase (*CYP17*), steroidogenic acute regulatory protein (*StAR*) and P450 scc (*CYP11*). The increased intracellular cAMP is known to associate with Protein Kinase A (PKA) holoenzyme, whose catalytic subunit translocates to the nucleus and phosphorylates the target proteins [16]. One of the well known and best studied targets of PKA is cAMP-responsive element-binding protein (CREB), which binds to the consensus CRE sequence found in the genome of certain cAMP-stimulated genes [17]. Phosphorylation of CREB Ser^{133} leads to the recruitment of the co-activator CREB-binding protein (CBP), which associates with the transcriptional machinery leading to increased gene transcription [18]. In humans and rat, the consensus CRE has an insertion of single base pair and is designated as cAMP-responsive element like sequence (CLS) [19]. However, the sequence has also been shown to bind the phosphorylated CREB protein and is a functional *cis*-element [20,21]. But in case of buffalo [7] and bovine [22] ovary, CLS has another point mutation (single base pair deletion) and hence is designated as CLS-like sequence. Hinshelwood et al. [22] has shown that this mutation in CLS in bovine ovarian *CYP19* gene promoter renders the sequence non-functional. No binding of CREB protein was seen to take place to this mutated CLS in bovine. Consequently, it was of interest for us to find out whether the observation made in bovine also pertains to buffalo CLS. Therefore, the present study was aimed to reveal the functional role of buffalo CLS-like sequence (buCLS) in *CYP19* gene regulation in ovary. In addition, experiments were also conducted to elucidate the probable regulatory molecules in addition to CREB as downstream effectors of cAMP/PKA pathway in regulation of *CYP19* gene in gonads.

Moreover, *in silico* analysis of buffalo ovary-specific promoter sequence [7] and experiments in other species, particularly rat and humans [23–25] showed the presence of several putative transcription factor binding sites that includes C/EBPβ, NR4A1/NUR77, SF1/NR5A1 and GATA family of transcription factors. Among these, C/EBPβ, NR4A1 and SF1 although are known to contribute to hormonal regulation of gonandal genes but also respond to several stimuli other than cAMP [13]. NR4A1 (NUR77) has been found to be upregulated by hCG/LH treatment in the rat and in the human granulosa tumor like cell line KGN [26,27]. The authors found that at least two members of NR4A orphan nuclear receptor family, NGFI-B & NR4A1 (NUR77) strongly inhibited transcription from ovary-specific promoter (PII) of the aromatase gene. Over expression of LRH-1 in rat granulosa cells does not enhance estrogen production or aromatase expression in either the presence or absence of FSH, although it did increase progesterone production [28]. This suggests that LRH-1 may not be crucial for aromatase expression in granulosa cells. Similarly, in normal ovaries, C/EBPβ mRNA is specifically induced by luteinizing hormone (LH/hCG) in the preovulatory antral follicles [27]. C/EBPβ has been shown to be essential for preovulatory granulosa cell differentiation in response to LH and thus was established as a critical downstream target of G-protein-coupled LH receptor signaling. C/EBPβ appears to act specifically as an LH-receptor responsive gene and may thus provide a tool to elucidate functional differences between the FSH and LH receptor systems [29]. The role of SF1 in the cAMP-dependent stimulation of certain gonandal genes has been well implicated. SF-1 is being constitutively phosphorylated *in vivo* by the mitogen-activated protein kinase (MAPK) pathway but its phosphorylation levels were not seen to be affected by cAMP treatment [30]. In steroidogenic tissues normally expressing SF-1, such as the placenta and skin, the expression of the cAMP regulated genes *CYP11A1* and *CYP17* was normal in SF-$1^{-/-}$ mice [31]. Thus, SF-1 might not be the major downstream effectors of cAMP signaling in gonandal cells.

Along with all the above-described factors, *CYP19* gene also contains the GATA motif for the binding of members of GATA family of transcription factors [7]. Of the six vertebrate GATA factors (GATA-1 to GATA-6), GATA-4 has been shown to be expressed in gonads and differentially activates the transcription of genes encoding steroidogenic enzymes. GATA-4 is strongly expressed from the onset of gonandal development and is later found in multiple cell lineages including testicular Sertoli and Leydig cells and granulosa cells of the ovary [32]. In gonads, GATA-4 has been shown to regulate the expression of several cAMP dependent gene promoters including StAR, inhibin-α and aromatase PII. Tremblay et al. [13,33] have shown that cAMP stimulation of gonandal cells leads to the phosphorylation of GATA-4 through PKA pathway and not the MAPK signal transduction pathway. The phosphorylation was seen to take place at Ser^{261} in mice which is an evolutionary conserved residue of GATA-4 protein. In rats GATA-4 binds to P450aromatase promoter and this binding is increased by $PGF_{2\alpha}$ [34]. Since the function of granulosa cells is under the hormonal control and with evidences of different species from literature supporting the role of GATA-4 in regulation of *CYP19* gene gonandal promoters, GATA-4 can be considered as a key downstream effector of cAMP/PKA pathway in addition to CREB in regulation of *CYP19* gene in buffalo ovary.

In view of the above, the present study was also aimed to delineate the role of transcription factor GATA-4 as the downstream effector of cAMP/PKA pathway in the absence of consensus CRE in buffalo ovary-specific *CYP19* gene promoter II (PII), the major ovarian promoter, during folliculogenesis and luteinization.

2. Materials and methods

2.1. Collection of buffalo ovaries and isolation of granulosa cells

Buffalo ovaries collection, follicular fluid aspiration, granulosa cells and postovulatory tissue (corpus luteum) isolation were done as described previously [9]. In brief, (approx 200) 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). Healthy, developing follicles were assessed by the presence of vascularized theca externa and clear amber follicular fluid with no debris. *In vivo*, follicular fluid was collected from small (≤5 mm) and large (≥8 mm) antral follicles while *in vitro* (cell culture) follicular fluid was collected from small and medium follicle (≤8 mm).

2.2. Preparation of reporter gene constructs, cell culture and transfection

2.2.1. Preparation of reporter gene constructs

Reporter gene constructs (deletion constructs) were prepared by PCR amplification of *CYP19* gene ovarian (*CYP19ov*) promoter II (PII) and PI.1 from the genomic DNA isolated from blood. Briefly,

Table 1
List of primer pairs used for amplification of *CYP19* gene promoter II and I.1.

Gene symbol	Primer sequence (5′–3′)	Size (bp)	Accession no.
With CLS	For GAACTCAGTCACTCTACCCACT	231	EU308111
CYP C	For GAACTCACGTCACTCTACCCACT	232	
Without CLS	For CCCACTCAAGGGCAAGGTGATA	211	
CYP 1R	Rev TGTTGCTTCAGAGGATGCTG		
PI.1	For CCTCACATTCCCTGACATCC	734	Z69241
	Rev TGCGTTGGCTCACCTACCT		

Table 2
Primer sequence used for PCR amplification of GATA-4.

Gene symbol	Primer sequence	Size (bp)	Accession no.
GATA-4	For 5′-AGGCCTCTTGCAATGCGGAAAG-3′ Rev 5′-GGACCTGCTGGTGTCTTTGATTTG-3′	86	NM_001192877
CYP19	For CCTGTGCGGGAAAGTACATCGC	105	DQ407274.2
	Rev TCTTCTCAACGCACCGATCTTG		

different primer pairs spanning different regions of PII (with and without CLS) were designed (Table 1). Primers were designed using Primer 3 software and NCBI tool. For the preparation of reporter construct containing buffalo CLS-like sequence with an inserted C, primer based insertion of the deleted cytosine in the bCLS sequence was made. The amplified PCR fragments were purified and cloned into the pCRII vector using the TA cloning system (Invitrogen). The cloned PCR products were custom sequenced by the ABI model 3730 sequencer using T7F and SP6R primers (Bangalore Genie, India) to ensure the fidelity of amplified fragments. The various deletion fragments were then subcloned into the KpnI and XhoI restriction sites in pGL3 basic vector. The fragments were cloned upstream of luciferase gene coding region (luciferase constructs). Reporter construct with cloned PI.1 was used as a negative control which was cloned in pCRII vector followed by sub cloning in pGL3 basic vector as described above for PII. The primers used for amplification of PI.1 are provided in Table 1.

2.2.2. Cell culture

All the culture reagents were purchased from Sigma Aldrich, Inc., St. Louis, USA unless otherwise stated. Cells were seeded in 24 well tissue culture plates (Nunc, Roskilde, Denmark) at a density of 2×10^5 viable cells in 1 ml of DMEM. For transfection of deletion constructs, the cells were initially cultured in medium containing serum for the first 24 h, in order to attain 80–90% cell confluency for transfection. After the first 24 h, the medium was replaced with serum free medium. The medium composition used was as follows: L-glutamine (3 mM), 3% FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml). Cultures were then maintained under serum free conditions at 37 °C in 5% CO_2, 95% air for 72 h, with 700 μl medium being replaced every 24 h. For siRNA transfection and gene expression analysis, the cells were cultured in serum free medium throughout the experiment with the following composition: L-glutamine (3 mM), protease free BSA (1 mg/ml), sodium selenite (4 ng/ml), transferrin (2.5 μg/ml), androstenedione (2 μM), bovine insulin (10 ng/ml), non-essential amino acid mix (1.1 mM), ovine FSH (1 ng/ml), human rIGF1 (1 ng/ml), penicillin (100 U/ml) and streptomycin (100 μg/ml). Cultures were maintained under serum free conditions at 37 °C in 5% CO_2, 95% air for 4 days (96 h), with 700 μl medium being replaced every 48 h (2 days). To analyze GATA-4 gene expression *in vitro*, following the first 48 h of cell attachment under serum free conditions, cells were either left untreated (control) or treated/stimulated with standardized doses of FSH (25 ng/ml) and IGF1 (50 ng/ml) in combination for 48 h. The cells were then lysed for RNA isolation and gene expression analysis. The primers used for GATA-4 gene expression analysis are listed in Table 2.

2.2.3. Transfection and Dual-Luciferase® reporter assay

1 μg of plasmid DNA was transfected in 24 well tissue culture plates in 50 μl DMEM per well. 2.5 μl of lipofectamine™ 2000 (Invitrogen, USA) per well was diluted in 50 μl DMEM lacking in serum and antibiotics and incubated at room temp for 5 min followed by gentle mixing and kept at room temperature in dark for 20–25 min to allow lipid–DNA complex formation. During the time of complex formation, medium from the culture plates was replaced with medium lacking serum and antibiotics thereby removing any remaining serum. Following complex formation, 1 ml of medium containing the lipid–plasmid DNA complex was then added to each well in culture plates and maintained at 37 °C and 5% CO_2 in air. Following 6 h of transfection, culture plates were taken out and the transfection medium was removed and the cells were either left untreated (control) or treated with medium containing ovine FSH (25 ng/ml) in combination with long R3 Insulin like Growth Factor I (50 ng/ml) for 18–24 h. Dose and time dependent studies of FSH and IGF1 on gene expression in serum free buffalo granulosa cell culture have been standardized in our laboratory [15]. The pGL3-control vector with SV40 promoter was used as a positive control of plasmid transfection and pRL-TK (Renilla-Thymidine Kinase) was used as an internal control. After 18–24 h, the cells were washed with PBS and lysed in Passive Lysis Buffer (PLB). For lysis, 25–30 μl of PLB was added to each well for lysis. The cell lysate were used immediately for estimation of promoter activity using reporter assay or stored at −80 °C until use. Dual-Luciferase® reporter assay was carried out as per the manufacturer's protocol using Dual-Luciferase® Reporter (DLR™) Assay System (Promega, Cat. # E1910). The reporter activity of firefly luciferase was normalized with renilla luciferase and the normalized activity was represented as Relative Luciferase Units (RLU).

2.3. Nuclear extracts and Western blots

In vivo, granulosa cells were isolated from different stages of folliculogenesis (small and large follicle) and luteinization (corpus luteum). Cells and tissue (CL) were washed with PBS containing protease inhibitor cocktail before lysis. 100 mg CL tissue was crushed in liquid nitrogen before lysis. Both cells and crushed tissue were lysed further, in 150–200 μl cell lysis buffer (HEPES, pH 7.5 (50 mM), KCl (10 mM), EDTA (1 mM), DTT (1 mM), 10% Triton X 100 and 1% protease inhibitor cocktail) and were incubated on ice for 10 min. *In vitro*, the cultured granulosa cells were either left untreated (control) or initially treated with FSH (25 ng/ml) + IGF1 (50 ng/ml) for 48 h. After treatment, cells were washed twice with ice-cold PBS and then cells were scraped in cell lysis buffer (with the above composition) and incubated on ice for 10 min followed by centrifugation at 12,000 rpm for 10 min at 4 °C. Removed the supernatant and added to the cell pellet the nuclear lysis buffer (HEPES, pH 7.5 (50 mM), KCl (10 mM), EDTA (1 mM), DTT (1 mM) and 1% protease inhibitor cocktail) and incubate the cells on ice for 30 min with intermittent vortexing. The nuclear lysate was then centrifuged at 14,000 rpm for 10 min at 4 °C to remove the cell debris and the supernatant was aliquoted and stored at −70 °C till further use. The protein estimation was done using Bradford assay.

Protein lysate (25 μg) were resolved by 10% SDS PAGE and then transferred to PVDF membrane (Paul corporation, cat# 66543) using the wet transfer Trans-Blot assembly (Bio-Rad). Membranes were blocked in Tris-buffered saline (TBS-T; 50 mM Tris, pH 7.4, 150 mM NaCl, 1% Tween-20) containing 5% (w/v) non-fat milk for 4 h at room temperature with gentle agitation. The membrane was then incubated overnight in TBS-TV (TBS-T; 50 mM Tris, pH 7.4, 150 mM NaCl, 1% Tween-20, Sodium Vanadate; 100 mM) with 1% non-fat milk containing antibodies to p-GATA-4 dilution 1:500 (Santacruz, cat# sc-32823) or non-phosphorylated GATA-4 dilution 1:500 (Santacruz, cat# sc-1237X) or β-actin dilution 1:500 (Santacruz, cat# sc-47778) with gentle agitation at 4 °C. After primary antibody incubation, the membranes were washed and incubated with horseradish peroxidase conjugated secondary antibodies (Santacruz, cat# sc-2350) in TBS-TV for 2 h at room temperature and then washed. A chemiluminescent signal was generated using enhanced chemiluminescence reagent (ECL™, Pierce) and membranes were exposed to X-ray film (Kodak biomax light film, Sigma). Housekeeping gene β-actin was used as a positive loading control.

2.4. Chemiluminescent electrophoretic mobility shift assay (EMSA)

Nuclear protein was isolated from both preovulatory (small and large follicles) and postovulatory (corpus luteum) stages of estrus cycle in the same manner as described in Section 2.3. The electrophoretic mobility shift assay was performed using Light shift® chemiluminescent kit (Pierce, Cat#20148) as per manufacturer's instructions with slight modifications. The probes were designed manually after *in silico* analysis of *CYP19* gene promoter II sequence and were labeled with biotin using biotin 3′-end labeling kit (Pierce, CAT# 89818). The oligonucleotides used for EMSA included annealed sense and antisense strands listed below (sense strand shown) – GATA-4: −269/−241, 5′-CAAGGGCAAGGTGATAAGGTTCTATCAGA-3′; buCLS: −299/267, 5′-TTAATTGAGAACTCAGTCACTCTACCCACTCAA-3′; hCLS: −299/267, 5′-TTAATTGAGAACTCACGTCACTCTACCCACTCAA-3′.

Protein binding reaction was carried out in a total volume of 20 μl consisting of 15 μg of total nuclear protein, 2 μl each of 10× binding buffer and Biotin end-labeled Target DNA (probe, 20 fmol). Further, 1 μl of 1 μg μl^{-1} poly(dI·dC) was added as a non-specific competitor and the reaction volume was made up to 20 μl with sterile water. Unlabeled probe at a concentration of 20 pmol/reaction was added as a cold competitor to one of the tube to check the specificity of the complex. No probe and no protein controls were prepared by adding all the components except labeled probe and nuclear protein, respectively. The reaction mixture was then incubated for 1 h in ice. The entire reaction was separated on 5% polyacrylamide gel in 0.5× Tris borate EDTA for 80 min at 4 °C. For supershift analysis, the samples (nuclear protein extracts) were first incubated with 1 μg of p-ser261 anti-GATA-4 antibody (Santacruz, cat# sc-32823) in ice for 30 min. Subsequently, all the other components, including probe, were added and incubated as described above. For CREB, biotinylated probes labeled at similar efficiencies were employed and the blots were exposed to film for the same length of time. The protein DNA complexes were transferred to nylon membrane using wet transfer trans-blot assembly (Bio-Rad). The membranes were then washed and incubated with streptavidin-horseradish peroxidase conjugate (1:300 dilutions) blocking solution for 1 h at room temperature with slight agitation. A chemiluminescent signal was generated using enhanced chemiluminescence reagent (Pierce, cat# 89800E&F) and membranes were exposed to X-ray film (Kodak biomax light film, Sigma).

Table 3
Primers for real time PCR amplification of immunoprecipitated DNA.

Gene	Primer sequence	Size (bp)
CYP19 gene promoter II	For 5′-GAACTCAGTCACTCTACCCACT-3′ Rev 5′-TGTTGCTTCAGAGGATGCTG-3′	231
GAPDH promoter	For 5′-CCGCATCCCTGAGACAAGA-3′ Rev 5′-GCCGTGGGTGGAATCATACT-3′	174

2.5. Chromatin immunoprecipitation (ChIP) assay

ChIP assay was carried out using EZ-Magna kit (Upstate Biotechnology, Cat. # 17-409), and as per the manufacturer's protocol. Briefly, *in vivo* experiments, formaldehyde (sigma) was added to granulosa cells isolated from small and large follicles at a final concentration of 1% for 10–15 min (room temp) to crosslink the DNA and associated proteins while corpus luteum (CL; 100 mg) tissue was crushed in liquid nitrogen followed by fixing with 1% formaldehyde. *In vitro*, the cells were initially treated with FSH (25 ng/ml) and IGF1 (50 ng/ml), in combination, for 48 h. The standardized conditions used for cell fixation, sonication, *etc.* were as described earlier [9]. 5 μg of phosphorylated GATA-4 (Ser 261) antibody was used for immunoprecipitation. Normal mouse IgG and no antibody were used as negative controls while Anti-RNA polymerase II antibody was included as a positive control. Purified DNA was then amplified using *CYP19* gene promoter specific primers while for positive control (anti-RNA polymerase), GAPDH promoter primers were used (Table 3).

2.6. RNA interference, RNA isolation, reverse transcription, real time PCR and ELISA

2.6.1. RNA interference

Cellular GATA-4 reduction was performed using siRNA designed from buffalo *CYP19* gene mRNA (accession no. JN165084) and were synthesized by Sigma–Aldrich. The sense and antisense strand sequences of GATA-4 used were: 5′-CCGGUCCUCUCCGCUCUGA-3′ and 5′-UCAGAGCGGAGAGGACCGG, respectively. Transfection of siRNA duplex was carried out in the same manner as stated in Section 2.2.3. Different concentrations of siRNA, *i.e.* 0 nM, 25 nM, 50 nM and 100 nM were used. Scrambled siRNA (cat # sc-37007, Santa Cruz, USA) was used as a negative control. Following transfection, 700 μl of medium was replaced with treatment medium containing ovine FSH (25 ng/ml) in combination with long R3 Insulin like Growth Factor I (50 ng/ml) for 12 h and 24 h each. Cells were lysed for RNA isolation for gene expression analysis and the culture medium from each of the control and experimental groups was collected for 17β-estradiol estimation using enzyme immuno assays.

2.6.2. RNA isolation

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

2.6.3. Real time PCR

Complimentary DNA was synthesized from 100 ng of total RNA using random hexamers and the First strand cDNA synthesis kit (Fermentas, Germany) following the manufacturer's instructions. 5 μl of prepared cDNA was amplified with SYBR Green master mix (Roche) in final reaction mixture of 12 μl using light cycler real time PCR (Roche Diagnostics, Mannheim, Germany). The amplification conditions were: pre-incubation at 95 °C for 5 min, followed by 40

cycles of denaturation 95 °C for 20 s, annealing at 60 °C for 15 s and extension at 72 °C for 15 s. Melting peaks were determined using melting curve analysis in order to ensure the amplification and thus generation of single product. Also, agarose gel electrophoresis analysis (1.5%) was carried out to determine the length of the amplified PCR product. Cloned PCR products for GATA-4 and *CYP19* gene were used to generate standard curves ranging over eight orders of magnitude (2.5×10^{-15} to 2×10^{-10} g DNA per reaction) using freshly diluted plasmids every time from the stock concentration of 10^{-9} g DNA/μl. Copy number was calculated relative to the amount of RNA that was subjected to cDNA preparation. PCR reaction with all the PCR reagents other than the added cDNA was used as a negative control. For ChIP assay, relative quantification method was used to calculate the fold enrichment of the gene of interest over non-immune serum control. Along with *CYP19* gene, both positive (GAPDH gene) and negative (IgG) controls were also amplified. BLAST analysis was done to check the specificity of the PCR reactions. Primers used were designed using Primer 3 software as listed in Table 2.

2.6.4. ELISA

The concentration of 17β-estradiol was determined using Enzyme Linked Immunosorbent Assay (ELISA) (Omega diagnostics, Scotland, UK). The 17β-estradiol standard curves were generated from the standards provided in the kit which ranged from 0 pg/ml to 1 ng/ml. Estimation was performed as per the manufacturer's instructions using 25 μl of spent medium for hormone estimation.

2.7. Data analysis

In real time PCR, fold enrichment was calculated using ΔΔCt method. ΔCt value of input DNA was used for normalization. ΔΔCt value was then calculated by subtracting the control values (negative control) from the ΔCt values of gene of interest. ΔΔCt values were converted to fold difference compared with control by raising two to the power ΔΔCt. Statistical analysis using Prism Software (Graph Pad Software, Inc., San Diego, CA, USA) was done. One-way analysis of variance (ANOVA) was used to estimate the statistical difference between different groups. ANOVA tests with a p value of ≤ 0.05 level were considered significant. All experimental data are presented as the mean ± SEM of three ($n = 3$) independent experiments. The correlation between GATA-4 and *CYP19* gene expression and 17β-estradiol contents was evaluated using a linear correlation model and statistical regression analysis.

3. Results

3.1. Buffalo CYP19ov/luciferase reporter activity

Reporter constructs used and luciferase assay have been shown in Fig. 1. In order to compare the promoter activity of buffalo CLS-like sequence with hCLS, a cytosine residue was inserted in buffalo CLS-like sequence. Reporter construct with cloned promoter I.1, which drives the residual expression of *CYP19* gene in the luteal tissue, was taken as negative control. The pGL3 control vector with SV40 promoter served as a positive control of plasmid transfection and showed very high luciferase activity between 350 and 450 relative luciferase units (not shown). The luciferase activity of deletion constructs of *CYP19*ov promoter both with and without CLS-like sequence was significantly higher ($p < 0.01$) than the basic vector and promoter I.1 (Fig. 1). However, no significant difference was seen to exist between the promoter activities of the two reporter constructs, with and without CLS-like sequence. The control wells (without treatment after transfection) showed less expression of promoter activity but the luciferase activity of cells treated with FSH (25 ng/ml) + IGF1 (50 ng/ml) was significantly higher ($p < 0.01$) in comparison to control. Difference in the promoter activity of the two constructs (with and without CLS-like sequence) in the control wells could be observed which might be due to the difference of two different reporter constructs of different DNA length. However, the difference between the two was not significant. Result showed that when transfection experiments were conducted using reporter

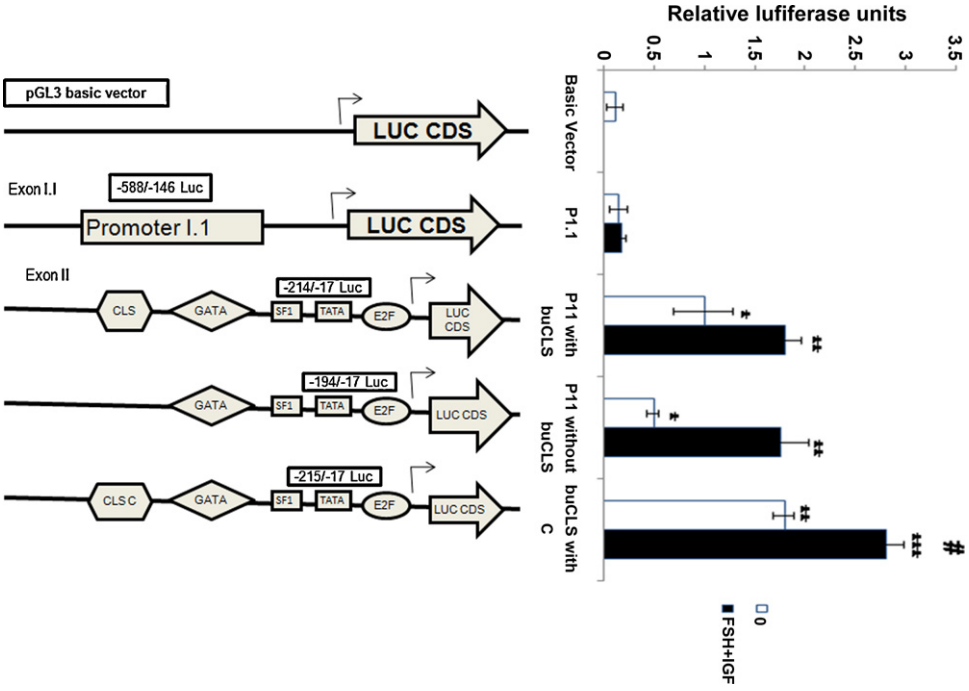

Fig. 1. Dual luciferase assay/reporter assay for assaying the activity of the respective PII reporter constructs. The activity of firefly luciferase was normalized with renilla luciferase (internal control) and expressed as Relative Luciferase Units (RLU). Treatment = FSH (25 ng/ml) + IGF1 (50 ng/ml). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, *vs* control (basic vector), $^{\#}p < 0.01$, significant difference of reporter construct with inserted "C" from reporter constructs with and without CLS-like sequence.

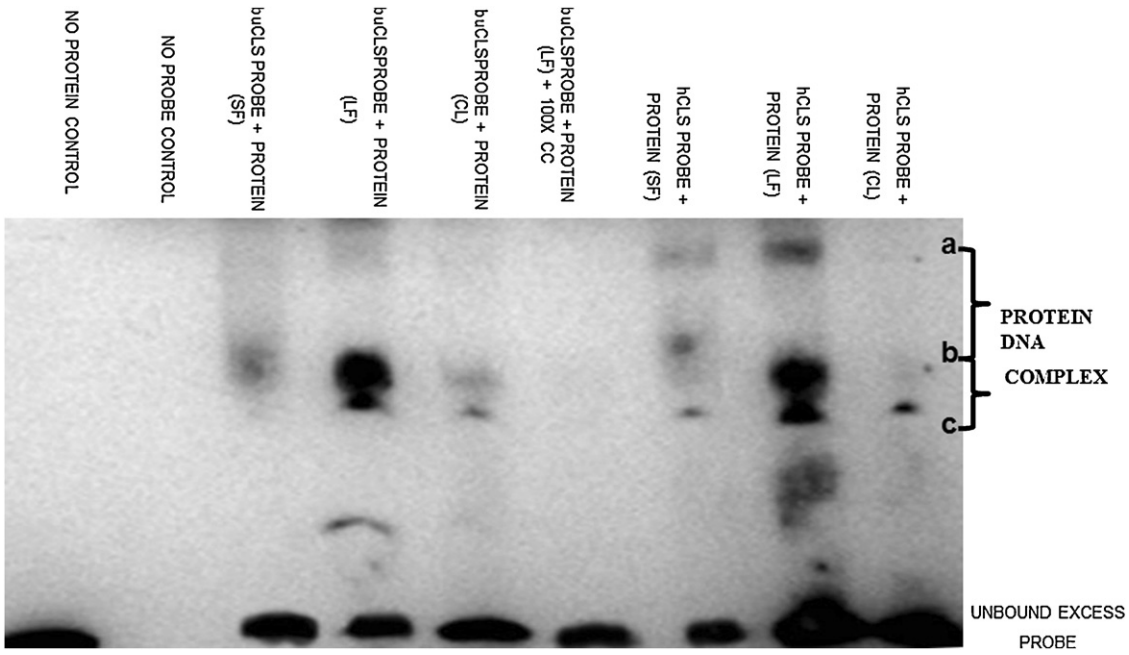

Fig. 2. EMSA studies for binding of the CREB transcription factor in different ovarian tissues. The binding was analyzed by incubating 15 fg biotin labeled PII specific probe with 15 μg of crude nuclear protein from granulosa cells of small follicle (SF), large follicle (LF) and corpus luteum (CL) on ice for 1 h. Shift was analyzed by running 5% native PAGE at 90 V for 80 min. For competition experiments, 100× unlabeled probe were added to the mix at the same time together with labeled probe. Both buffalo and human PII probes were used for shift assays.

construct (−215/−17) with an inserted C which corresponds to the human CLS, the luciferase activity was seen to increase significantly ($p < 0.01$) in comparison to other two reporter constructs. Since no difference in reporter activity was seen between the PII deletion constructs (with and without CLS-like sequence) while a sharp increase was observed for reporter construct with inserted cytosine in CLS-like sequence.

3.2. Low affinity nuclear protein–DNA complexes of buffalo CLS

To test further the functional aspects of buffalo CLS-like sequence, an electrophoretic mobility shift assay (EMSA) experiment was performed. The nuclear protein was isolated from both preovulatory (granulosa cells small and large follicles) and postovulatory (corpus luteum) stages of buffalo ovary. The isolated protein was incubated with biotinylated oligonucleotides that correspond to the CLS sequences from buffalo and human *CYP19*ov 5′ flanking sequences. The two sequences differ only in 1 bp (cytosine) which is deleted in buffalo CLS. Fig. 2 depicts the EMSA experiment results. No protein and no probe were used as negative controls for protein and probe respectively. Result showed that when the buffalo CLS-like sequence was used as probe, a binding pattern consisting of two major complexes (b and c) was observed. Each of the complexes was effectively competed with 100-fold molar excess of non-labeled buffalo CLS probe. However, incubation of protein with human CLS (hCLS) showed the formation of three complexes (a, b and c). The intensity of the complex 'a' in human was the maximum in large follicle in comparison to other tissues but was found to be absent in buffalo. The complex 'a' has been reported to be the only functional complex (as will be referred in Section 4.1) formed after the binding of CREB to human CLS. Thus as the buffalo CLS-like sequence does not form functional DNA nuclear protein complex 'a' (compared to hCLS), it can be concluded that buffalo CLS-like sequence cannot interact with proteins unlike the human CLS. The intensity of complex 'a' (in human) was found to be maximum with the nuclear protein isolated from the granulosa cells aspirated from large follicle.

3.3. Expression of GATA-4 mRNA

The transcript abundance of GATA-4 gene was quantified using real time PCR. Fig. 3A shows the absolute copy number of GATA-4 transcripts during different stages of folliculogenesis and luteinization. GATA-4 transcript present in granulosa cells of large follicle was significantly higher ($p < 0.01$) in comparison to small follicle granulosa cells and corpus luteum. *In vitro* (Fig. 3B), transcript abundance of GATA-4 gene was seen to be significantly higher ($p < 0.001$) in the treated cells (FSH 25 ng/ml + IGF1 50 ng/ml) in comparison to control (untreated) cells.

3.4. Phosphorylated GATA-4 protein binds to buffalo CYP19 gene ovarian promoter

In order to find out whether GATA-4 protein binds to *CYP19* gene ovarian promoter and whether any difference exists in it's binding in the preovulatory and postovulatory stages of buffalo ovary, both EMSA and ChIP experiments were performed. For EMSA, the nuclear protein isolated from both preovulatory (granulosa cells from small follicles and large follicles) and postovulatory (corpus luteum) stages was incubated with biotinylated oligonucleotides that correspond to the GATA sequences from buffalo *CYP19*ov 5′ flanking sequences. The results showed (Fig. 4) that shift was observed in all the stages of estrus cycle. Though, the band intensity was observed to be the maximum in corpus luteum, preincubation of nuclear extracts with phosphorylated (Ser261) GATA-4 antibody did change the intensity of complex formed in corpus luteum (CL) while the intensity of the complex formed with nuclear protein isolated from granulosa cells of large follicles was drastically reduced. It can be inferred from the above result that some non-specific complex is being formed in CL whose intensity did not change with the addition of specific phosphorylated GATA-4 antibody while in large follicle the decline in band intensity signifies the formation of a specific complex.

To validate and confirm, further, the binding of phosphorylated GATA-4 protein to *CYP19* gene promoter, ChIP assay was

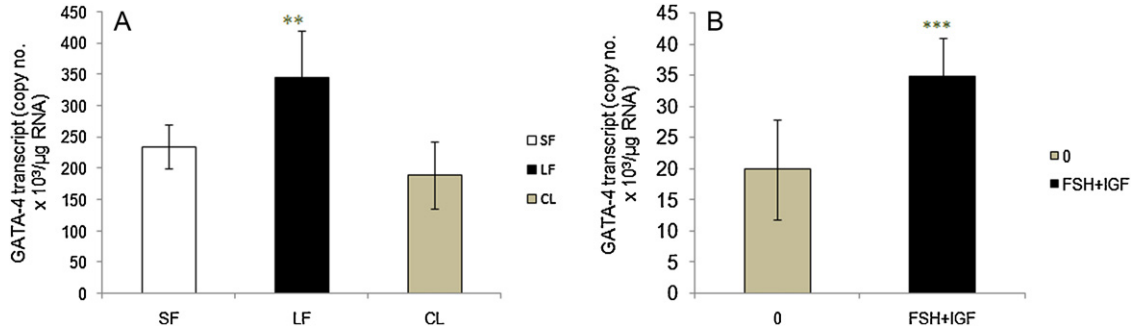

Fig. 3. Relative abundance of GATA-4 transcripts in granulosa cells from different stages of folliculogenesis and differentiation under *in vivo* conditions and its regulation by FSH and IGF-I in serum free cultured buffalo granulosa cells under *in vitro* conditions: (A) expression of GATA-4 in granulosa cells of small follicle (SF), large follicle (LF) and corpus luteum (CL). GATA-4 transcripts present in granulosa cells of large follicle were significantly higher ($p < 0.01$) in comparison to small follicle granulosa cells and corpus luteum. (B) In serum free buffalo granulosa cells culture (*in vitro*), copy numbers of GATA-4 mRNA were seen to be significantly higher in the treated cells (FSH 25 ng/ml + IGF1 50 ng/ml). Values are represented as means ± SEM of three independent experiments. Copy number was calculated and normalized to initial amount of RNA used for cDNA preparation. $^{**}p < 0.01$, LF *vs* SF and CL, $^{***}p < 0.001$, *vs* control.

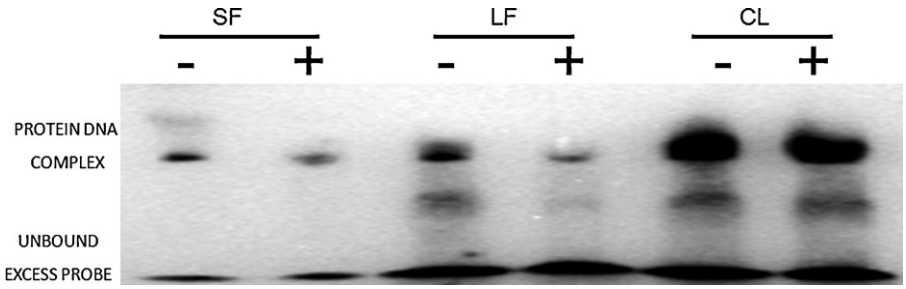

Fig. 4. EMSA studies for binding of transcription factor GATA-4 in different ovarian tissues. The binding was analyzed by incubating 15 fg biotin labeled PII specific probe with 15 μg of crude nuclear protein from granulosa cells of small follicle (SF), large follicle (LF) and corpus luteum (CL) on ice for 1 h. Shift was analyzed by running 5% native PAGE at 90 V for 80 min. For supershift experiments 1 μg of anti ser p-261 GATA4 antibody was added 30 min before adding the labeled probe (+ indicates Ab is added; – indicates the absence of Ab).

performed. Table 4 gives the Ct values obtained from amplification of immunoprecipitated DNA. The results of ChIP assay (Fig. 5) were concurrent to EMSA. Significant difference was seen to exist for binding of phosphorylated form of GATA-4 protein between large follicle granulosa cells ($p < 0.001$) and luteal tissue (Fig. 5A). In granulosa cells of large follicle, ~25-fold higher binding of GATA-4 to *CYP19* gene ovarian promoter (PII) was observed in comparison to luteal tissue. However, the difference was only ~3-fold in comparison to binding observed in small follicles. Similar results were also obtained under *in vitro* conditions (Fig. 5B). Treated cells (FSH 25 ng/ml + IGF1 50 ng/ml) were seen to have ~35-fold higher binding of phosphorylated GATA-4 protein in comparison to control (non-treated). Comparison of the real time PCR results using DNA from the sample obtained from DNA protein complex incubated with the mouse IgG (negative control) and phosphorylated (Ser-261) anti-GATA-4 antibody obtained from granulosa cells of large follicle and in treated cultured granulosa cells (*in vitro*) showed that the phosphorylated (Ser261) anti- GATA-4 antibody was able to pull down ~250- and ~70-fold more DNA, respectively, as compared to respective negative controls. These results clearly demonstrated that one of the reasons for the loss of *CYP19* gene PII activity in buffalo corpus luteum could be the lack of binding of regulatory molecule phosphorylated GATA-4. Thus, GATA-4 appears to be an important *trans*-acting molecule binding to *CYP19* gene promoter and can be involved in regulating its expression during folliculogenesis and luteinization.

3.5. Effects of GATA-4 mRNA knockdown on CYP19 gene expression and 17β-estradiol content

The next aim of the experiment was to verify the effect of GATA-4 knock down on *CYP19* gene expression and 17β-estradiol production. To address this question siRNA were designed against the GATA-4 mRNA and transfected in cultured buffalo granulosa cells. Following transfection, the cells were treated with FSH (25 ng/ml) and IGF1 (50 ng/ml) in combination. Cell cultures were terminated after 12 and 24 h treatment and analyzed for GATA-4, *CYP19* gene expression and hormone (17β-estradiol) production.

Table 4
Quantitative real time PCR results (Ct values). Ct values >35 had no detectable *CYP19* gene promoter.

Cell type (buffalo ovarian cells)	ChIP assay: proximal (PII) of *CYP19* gene promoter				No antibody (−ve control)	IgG (−ve control)
	Promoter	Anti-GATA 4 (S261)	DNA input	Anti-RNA polymerase (+ve control)		
Small follicle	Proximal	27–29	18–20	23–25	>35	37–40
Large follicle	Proximal	21–23	17–19	22–25	>35	35–38
Corpus luteum	Proximal	27–29	18–20	22–24	>35	34–37
Cultured cell						
Control	Proximal	32–35	31–34	24–26	>35	38–40
Treatment		28–30	30–32			

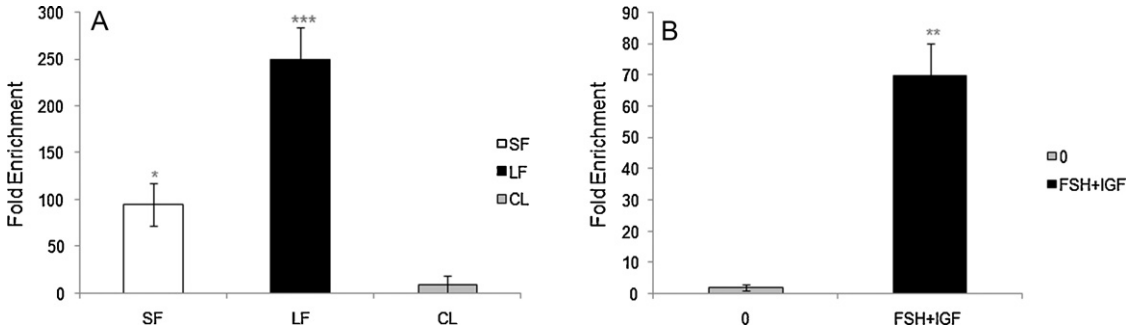

Fig. 5. Analysis of GATA-4 binding to *CYP19* gene promoter (PII) in buffalo granulosa cells during different stages of folliculogenesis, differentiation *in vivo* and cultured granulosa cells *in vitro*. Chromatin immunoprecipitation (ChIP) assay was performed using phosphorylated GATA-4 (S261) antibody: (A) significant difference was observed for phosphorylated GATA-4 (S261) binding in promoter (PII) of *CYP19* gene in granulosa cells of large follicles (LF) (~25-fold higher binding in LF in comparison to corpus luteum (CL) while difference was only ~3-fold when compared to granulosa cells of small follicles (SF)). (B) Under *in vitro*, granulosa cells were cultured as described in details in materials and methods and treated (FSH 25 ng/ml + IGF1 50 ng/ml). ~35-fold higher binding of phosphorylated GATA-4 protein was observed in treated cells in comparison to control (non-treated). Relative binding/fold enrichment was quantified using real time PCR. The ChIP data was normalized to starting (input) DNA and the results were expressed as fold enrichment over the non-immune control antibody, Values are represented as means ± SEM of three independent experiments. (A) $^{*}p < 0.05$, SF *vs* CL, $^{***}p < 0.001$, LF *vs* CL; (B) $^{**}p < 0.01$, *vs* control, Treatment = FSH (25 ng/ml) + IGF1 (50 ng/ml).

Scrambled siRNA was used as a negative control. Results showed significant knockdown of GATA-4 mRNA (Fig. 6A) when transfected with 50 nM ($p < 0.05$) and 100 nM ($p < 0.001$) siRNA after 24 h of gonadotropin treatment. No significant knockdown was observed at 12 h. Absolute quantification of *CYP19* gene transcript (Fig. 6B) revealed a sharp and significant decline concurrent to GATA-4 at 25 nM ($p < 0.01$), 50 nM ($p < 0.001$) and 100 nM ($p < 0.001$) siRNA concentration. Similarly, no significant knockdown of *CYP19* gene expression was observed at 12 h. Measurement of 17β-estradiol content using ELISA (Fig. 6C) showed a decline in 17β-estradiol content at 50 nM ($p < 0.01$) and 100 nM ($p < 0.001$) siRNA concentration at which *CYP19* gene expression was also drastically reduced. With the knockdown of GATA-4 transcript, a concurrent decline was observed in *CYP19* gene expression which was followed by a sharp decline in 17β-estradiol production. Scrambled siRNA used as a negative control did not cause any significant decline in gene expression and hormone content. GATA-4 RNAi effectively reduced GATA-4 mRNA by 53% and 81% after 24 h at 50 nM and 100 nM siRNA concentrations respectively. While, percentage knockdown of *CYP19* message achieved at 50 nM and 100 nM siRNA concentration was 83% and 85% respectively. Thus, decrease in GATA-4 mRNA significantly ($p < 0.001$) reduced the transcript abundance of *CYP19* gene and consequently significant ($p < 0.001$) decline in 17β-estradiol contents was also observed.

3.6. GATA-4 is phosphorylated at Ser261 following stimulation with gonadotropins

Phosphorylation of GATA-4 protein was investigated using Immunoblotting. Both unphosphorylated and phosphorylated

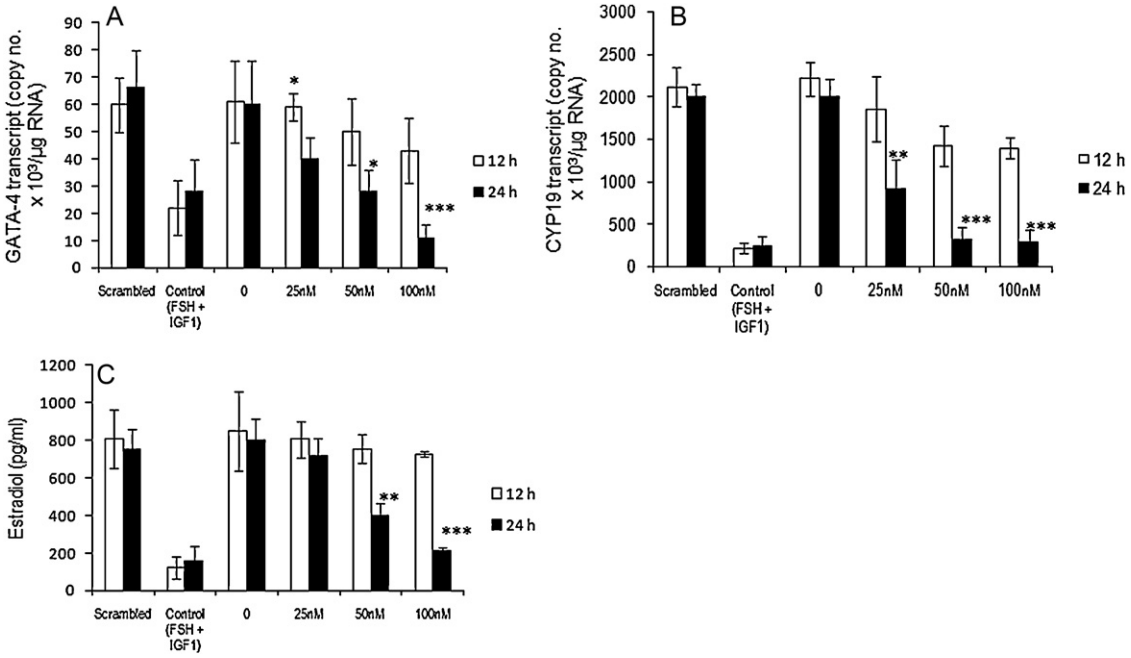

Fig. 6. Effects of GATA-4 silencing on *CYP19* gene expression and 17-β-estradiol content: (A) absolute quantification of GATA-4, (B) *CYP19* gene transcripts and (C) 17-β-estradiol contents was measured following GATA-4 siRNA transfection in buffalo serum free granulosa cell culture. Cells were cultured for 96 h under complete serum free conditions. After the initial 72 h of culture, cells were transfected with different conc. of siRNA (0, 25, 50 and 100 nM). Following 6 h of transfection, the cells were treated with FSH (25 ng/ml) + IGF1 (50 ng/ml) for 12 and 24 h. Isolated RNA was reverse transcribed and amplified by SYBR green real time PCR. Copy number was calculated and normalized to initial amount of RNA used for cDNA preparation. GATA-4 values are means ± SEM of three independent cultures. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, *vs* control. Scrambled siRNA used as a negative control did not cause any significant decline in gene expression and hormone content.

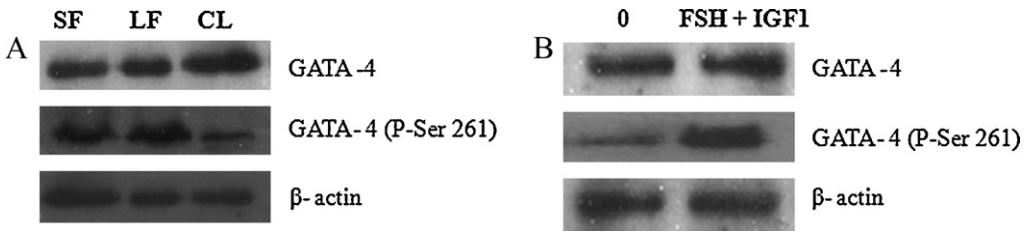

Fig. 7. Relative abundance of GATA-4 protein in granulosa cells from different stages of folliculogenesis, differentiation *in vivo* and its regulation by FSH and IGF-l in serum free cultured buffalo granulosa cells *in vitro:* Western immunoblotting analysis was performed with nuclear protein isolated from granulosa cells of different stage of folliculogenesis (SF, granulosa cells of small follicle; LF, granulosa cells of large follicle) and differentiation (CL, corpus luteum) (A) and cultured granulosa cells (B) as described in materials and methods. Both phosphorylated (P-Ser261) and unphosphorylated forms of GATA-4 antibodies were used. β-Actin was used as a positive loading control. The immunoblot shown here is the representative of three separate experiments.

GATA-4 antibodies were used for detection of GATA-4 protein. *In vivo*, the band intensities of unphosphorylated GATA-4 protein were the same in all the stages of estrus cycle (Fig. 7A) and similar results were obtained also *in vitro* (Fig. 7B). However, western blot using phosphorylated GATA-4 (Ser261) protein showed differential expression during different stage of folliculogenesis and luteinization. The intensity of phosphorylated GATA-4 protein was found to be higher in protein isolated from granulosa cells of large follicle in comparison to small follicle granulosa cells nuclear protein and corpus luteum. Similarly, *in vitro*, maximum phosphorylated GATA-4 protein was observed in the FSH and IGF1 treated cells as compared to respective control. β-Actin was used as a positive loading control.

4. Discussion

4.1. Buffalo CLS-like sequence with a point mutation is not a functional cis-element

The ovary specific 5′ flanking regions and promoters of *CYP19* gene from different species show high homology in the conserved element sequences [22]. However, there are still some marked differences which can be seen as point mutations in certain *cis*-acting elements. The regulatory sequence, cAMP-response element (CRE: 5′-TGACGTCA-3′) that binds the cAMP-response element binding protein (CREB), has a single mutation in humans and rat and is designated as cAMP-response element-like sequence (CLS): 5′-TGcACGTCA-3′. This element is shown to be critical for cAMP stimulated transcription of human and rat ovarian *CYP19* gene [19,35]. However, in cattle [22] and buffalo [7], the *CYP19* gene has a single base pair deletion in a cAMP-response element-like sequence (CLS) and is designated as CLS-like sequence. Reporter assay results showed that although significant difference exists between *CYP19* gene promoter II activity and pGL3 basic vector ($p < 0.01$), no difference was observed between the promoter activities of reporter constructs with and without CLS-like sequence. However, a significant ($p < 0.01$) increase in promoter activity occurred after insertion of cytosine in buffalo CLS-like sequence (−215/−17) which corresponds to human CLS. The above results are supported by the studies carried out in bovine. Hinshelwood et al. [22] have shown that mutation of bovine CLS to human CLS causes the bovine construct to acquire the cAMP responsiveness but the luciferase activity never matched the wild type human CLS reporter construct. It was suggested that CLS-like sequence is non-functional and some other *cis*-acting elements in this region are involved in *CYP19* gene regulation.

In the present study IGF1 was used along with gonadotropins since growth factors like IGF1 have been reported to be critical intrafollicular factor which promote increased responsiveness of the dominant follicle to gonadotropins (FSH) at the time of follicle selection [36]. Also IGF1 has been described to synergize the effects of FSH on *CYP19* gene expression in bovine [14] and buffalo [15] cultured granulosa cells.

Binding studies of transcription factor CREB to CLS-like sequence in buffalo *CYP19* gene promoter II was done using EMSA. Analysis of CREB binding to buffalo CLS using EMSA showed that buffalo CLS-like sequence formed two complexes (b and c) whereas the human probe was seen to form three complexes (a–c) when incubated with granulosa cells nuclear protein of large follicle. These results were obtained when probes biotin labeled at similar efficiencies were employed and the blots were exposed to film for the same length of time. In rat and human luteal cells, also in rabbits, estrogen synthesis is observed [37,38]. This does not seem to be the case for bovine and buffalo corpora lutea. In human [22] and rabbit [23], three complexes were observed. The complex a is reported to be due to the binding of hetero-dimer of CREB-A and CREB-B proteins [39] and was found to be the only functional complex responsible for *CYP19* gene expression in rat granulosa cells [31]. It, therefore, appears as if through evolution, the buffalo has lost a *cis*-acting element important for cAMP-induced transcription of the *CYP19*ov gene in other species. Our results coincide with the earlier studies in bovine which showed almost similar results when human CLS and bovine CLS were incubated with nuclear extracts of cultured bovine granulosa cells as stated above. To the best of our knowledge, this is the first report of species-specific differences in CREBP binding to CLS of *CYP19ov* gene *in vivo*. The authors above [22] also proposed that the non-functionality of CLS might be responsible for loss of *CYP19* gene expression after luteinization. But this possibility was ruled out by further experiments carried out in different species. For example, in rat, Stocco et al. [21] suggests that the control of *CYP19* gene promoter activity in the luteal cells is not dependent of CLS but it seems to be dependent of GATA, NRE and AP3 sites. Surprisingly, in Rhesus monkey corpus luteum/corpora lutea, PKA activity is maintained, but the expression of CREB is decreased after ovulation and during luteinization [40]. In rabbit, expression of CREB and its binding on CLS have been shown in luteal cells and were found to be the major cAMP responsive element maintained throughout the luteal phase within the PII region. Therefore, additional sites other than the CLS-like sequence are necessary in the post-LH surge down-regulation of *CYP19* gene transcription in rabbit due to persistence in functionality of CLS [35]. The above authors also stated that although the NREA site is involved in the regulation of aromatase expression in rat granulosa and luteal cells, this site is not necessary to activate the PII in response to cAMP in rabbit granulosa and luteal cells. In the very same way, in the absence of functional CLS in buffalo *CYP19* gene ovarian promoter, certain other *cis*-acting elements downstream of the CLS in this region might be necessary for mediating the cAMP responsiveness of *CYP19* gene in buffalo ovary. Therefore, although cAMP is the major regulator of the proximal promoter of aromatase across species, the elements required for the expression of aromatase differs between species.

4.2. Phosphorylated GATA-4 is the downstream effector of PKA signaling pathway regulating ovarian CYP19 gene expression in buffalo

Once it was verified that buffalo CLS is not a functional *cis*-acting element and is playing no significant role in regulating *CYP19* gene expression in ovary, the next goal was to identify other probable sequences in the vicinity of CLS that can bind to *trans*-acting molecules and thereby regulate gene expression. As stated in section I, *CYP19* gene promoter II has the binding sites for several transcription factors which have been described as important cAMP responsive factors in different species. GATA-4 is an essential ovary specific transcription factor whose role in *CYP19* gene regulation has been well established in the rat ovary [13]. We investigated the involvement of GATA-4 as a downstream effector of PKA pathway in buffalo ovary in the absence of consensus CRE sequence and thus in the regulation of *CYP19* gene expression. Efforts were made to understand its possible involvement both *in vivo* and *in vitro*. *In vitro*, granulosa cells were cultured under serum free conditions for 96 h. Since maintenance of *CYP19* gene expression in culture conditions with serum is difficult as granulosa cells have the tendency to luteinize [41,42] and loose their granulosa cell phenotype along with *CYP19* gene activity [43] in presence of serum, a serum free cell culture system was developed. However, for reporter construct transfection, cells were cultured initially for 24 h with serum for attaining 80-90% cell confluency for transfection.

Result showed that, *in vivo*, the expression of GATA-4 was significantly higher in granulosa cells of large follicle where maximum *CYP19* gene expression occurred. Similarly, the expression significantly increased in treated cells (FSH 25 ng/ml + IGF1 50 ng/ml) as compared to control (untreated) cells *in vitro*. Thus the expression of GATA-4 gene was observed to be FSH responsive. In humans, ovarian tissue samples and freshly isolated granulosa-luteal cells derived from preovulatory follicles of gonadotropin-treated women express GATA-4 mRNA and also GATA-4 protein [44]. However, specie- and tissue-specific effects of GATA-4 have been identified. GATA-4 and C/EBP β are both required for FSH + IGFI stimulation of the porcine steroidogenic acute regulatory protein gene promoter in homologous granulosa cell cultures [45]. GATA-4 reduction has been shown to enhance cAMP stimulated steroidogenic acute regulatory (*StAR*) protein mRNA and progesterone production in luteinized porcine granulosa cells [46]. During the postovulatory luteinization period, GATA4 levels have been shown to decline and remain low in corpora lutea [47–49].

Consequently, our next interest was to find out whether the phosphorylated form of GATA-4 protein binds to buffalo ovary specific *CYP19* gene promoter. Both EMSA and ChIP assay results showed significantly increased binding of phosphorylated GATA-4 protein to *CYP19* gene ovarian promoter when isolated from granulosa cells of large follicle in comparison to granulosa cells protein of small follicle and luteal tissue. In the rat CL, dramatic changes in P450arom mRNA levels takes place throughout pregnancy. In this species it was reported that GATA-4 is expressed in the CL at the end of pregnancy, binds to P450arom promoter and such binding is increased by $PGF_{2\alpha}$ treatment which attenuates *CYP19* gene expression [34]. Also, in 2007, Stocco et al. [21] have shown that along with GATA, AP3 and NRE sites are associated with changes in *CYP19* gene expression in rat luteal cells. To validate further the role of GATA-4 gene as *trans*-acting element regulating *CYP19* gene expression, siRNA designed against the GATA-4 mRNA were transfected in cultured granulosa cells and the transcript abundance of GATA-4 and *CYP19* gene were quantified along with 17β-estradiol content estimation. Results showed that knockdown of GATA-4 mRNA significantly declined *CYP19* gene expression and a concurrent decline in 17β-estradiol content was also observed. However, in the present study, close association between knockdown of *CYP19* gene transcript and the reduction in 17β-estradiol contents was not observed for 25 nM of siRNA transfection. Literature has evidences for the same. 17β-Estradiol concentrations declined significantly before P450arom mRNA levels were known to decline [50]. Significant association between the expression of P450arom mRNA and aromatase protein activity in human breast tumors was not observed [51]. One probable reason for the same can be gene regulation of *CYP19* gene by gonadotropins which can modulate aromatase activity both at transcription and translation/enzyme levels. Other can be the production of 17β-estradiol from the extant enzyme remaining after the aromatase mRNA suppression. Hui and LaVoie [46] have explored the effect of GATA-4 knockdown using RNAi on *StAR* gene expression. They had also found that the decrease in GATA-4 transcripts significantly stimulated *StAR* gene expression in porcine luteinized granulosa cells.

We now aimed to explore whether PKA mediated phosphorylation (Ser261) of GATA-4 protein is related to the FSH responsive expression of GATA-4 gene and its binding in the phosphorylated form to the *CYP19* gene ovarian promoter. Western blot experiments were done using both phosphorylated and unphosphorylated antibodies against GATA-4 protein. As hypothesized, results showed that unphosphorylated GATA-4 was present in same amount in all the stages of estrus cycle *in vivo* and no change in unphosphorylated protein concentrations was observed between control and treated cells *in vitro* as well. However, phosphorylated GATA-4 (Ser261) was the maximum in large follicle and also in the treated cells (FSH 25 ng/ml + IGF150 ng/ml) as compared to control (untreated) cells *in vitro*. As both the FSH responsive GATA-4 transcript abundance and phosphorylated protein were the maximum in large follicle and FSH 25 ng/ml + IGF1 50 ng/ml treated cells, it was inferred that GATA-4 could be a downstream effector of PKA pathway and thus is involved in the regulation of the *CYP19* gene expression in buffalo ovary as also FSH mediated PKA pathways is well established and exist in gonandal cells [13,14,33]. Salvador et al. [17] have shown in rat granulosa cells that phosphorylated CREB recruits and bind the coactivator protein CBP. CBP is known to bind not only to phospho-CREB but also to SF1 [52,53] and enhance expression of SF1 regulated genes [53]. The CBP in turn increases the histone acetyltransferase activity by recruiting HATs such as PCAF (CBP-associated factor) [17,54,55]. Furthermore, GATA-4 is predominantly phosphorylated on serine residue located at position 261 by PKA in response to cAMP in gonandal cells [19]. The above authors have also shown that PKA dependent phosphorylation of GATA-4 also led to enhanced recruitment of the CREB-binding protein (CBP) coactivator. Consequently, it is possible that in the absence of consensus CRE in buffalo, instead of CREB, phosphorylated GATA-4 binds to *CYP19* gene promoter, recruits CBP which in addition engage histone acetylase (HATs). In the luteal tissue, no binding of GATA-4 protein takes place to *CYP19* gene promoter II which is accountable for the down regulation of *CYP19* gene expression after luteinization. It can be considered that certain inhibitory factors might be preventing the binding of GATA-4 to ovary-specific *CYP19* gene promoter (PII); consequently, PII is switched off resulting in decline in *CYP19* gene expression in the luteal tissue. Therefore, it can be established that in the buffalo ovary, the catalytic subunit of PKA following stimulation with FSH and IGF1 translocates to the nucleus, phosphorylates GATA-4 which binds to PII, can also form complex with other transcription factors, recruit CBP and possibly PCAF which acetylates histone (H3), facilitating the opening of chromatin structure, engagement of basal transcription machinery and initiation of transcription (Fig. 8). In our previous study [9], we have shown that chromatin remodeling through histone modifications have an important role in regulating the promoter switching mechanism of region I.1 (PI.1) of *CYP19* gene and not PII. Consequently, it is possible that GATA-4 instead of histone modifications is regulating the PII activity and thus the

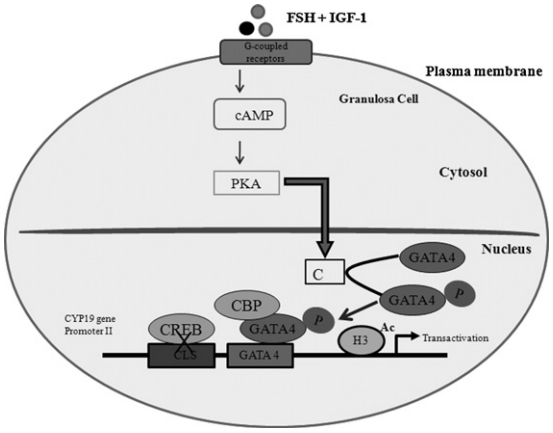

Fig. 8. Proposed model for FSH signaling in regulation of *CYP19* gene in buffalo ovary. The catalytic subunit of PKA will catalyze the phosphorylation of GATA-4 other than CREB. Phosphorylated GATA-4 binds to *CYP19* gene promoter II and possibly in conjunction with other transcription factors, recruits coactivator proteins like CBP and also histone acetyl transferase (HATs), induces histone acetylation and gene transactivation.

differential expression of *CYP19* gene during folliculogenesis and luteinization.

In conclusion, the present study demonstrated the involvement of GATA-4, an essential transcription factor and a downstream effector of PKA pathway, in regulation of *CYP19* gene promoter during folliculogenesis and luteinization in buffalo ovary. Also we have presented the first report on the *in vivo* binding of phosphorylated GATA-4 to ovary-specific *CYP19* gene promoter (PII) during the estrus cycle. This is the first study which elucidated the involvement of GATA-4 *trans*-acting molecule as a downstream effector of PKA pathway in *CYP19* gene regulation in the absence of consensus CRE in ruminants.

Acknowledgements

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