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# Validation of Gene Silencing Using RNA Interference in Buffalo Granulosa Cells

**Rachna Monga, Tirtha Kumar Datta[1] and Dheer Singh***

Molecular Endocrinology Laboratory, Animal Biochemistry Division, Animal Biotechnology Center[1],
National Dairy Research Institute, Karnal- 132001, Haryana, India

**ABSTRACT :** Silencing of a specific gene using RNAi (RNA interference) is a valuable tool for functional analysis of a target gene. However, information on RNAi for analysis of gene function in farm animals is relatively nil. In the present study, we have validated the interfering effects of siRNA (small interfering RNA) using both quantitative and qualitative gene silencing in buffalo granulosa cells. Qualitative gene knockdown was validated using a fluorescent vector, enhanced green fluorescence protein (EGFP) and fluorescently labeled siRNA (Cy3) duplex. While quantitatively, siRNA targeted against the luciferase and *CYP19* mRNA was used to validate the technique. *CYP19* gene, a candidate fertility gene, was selected as a model to demonstrate the technique optimization. However, to sustain the expression of *CYP19* gene in culture conditions using serum is difficult because granulosa cells have the tendency to luteinize in presence of serum. Therefore, serum free culture conditions were optimized for transfection and were found to be more suitable for the maintenance of *CYP19* gene transcripts in comparison to culture conditions with serum. Decline in fluorescence intensity of green fluorescent protein (EGFP) was observed following co-transfection with plasmid generating siRNA targeted against EGFP gene. Quantitative decrease in luminescence was seen when co-transfected with siRNA against the luciferase gene. A significant suppressive effect on the mRNA levels of *CYP19* gene at 100 nM siRNA concentration was observed. Also, measurement of estradiol levels using ELISA (enzyme-linked immunosorbent assay) showed a significant decline in comparison to control. In conclusion, the present study validated gene silencing using RNAi in cultured buffalo granulosa cells which can be used as an effective tool for functional analysis of target genes. (**Key Words :** RNAi, Transfection, *CYP19* Gene, 17β- Estradiol, Buffalo, Transcript Abundance)

## INTRODUCTION

Discovery of RNAi, both as a target and mediator of controlling gene expression has now become well apparent. Although, for the functional analysis of any gene, a variety of tools are known including mutagenesis, transgenic techniques or gene knockouts but still these techniques are technically challenging in ruminants (Hirano et al., 2004). Exploring gene function using these techniques is not only time consuming but also laborious and expensive than silencing genes by merely using double stranded oligonucleotides about twenty residues in length (Fire and Nirenberg, 2005). Therefore, these approaches are now being superseded by RNA interference (RNAi), the biological mechanism by which double stranded RNA (dsRNA) induces gene silencing by targeting complementary mRNA and thereby causing its degradation (Aravin et al., 2001; Khatri et al., 2006). RNAi technology is technically simple, quick and no initial knowledge of the entire sequence of a gene or its flanking sequences is required (Brüning-Richardson and McConkey, 2005). However, the stability, transfection, and knockdown efficiency of siRNA are influenced by various factors like cell type (Guerra-Crespo et al., 2003), cell number, transfection reagents (Gonzalez et al., 2007) and type of culture.

A key endocrine marker of differentiating ovarian granulosa cells is the ability to synthesize estrogens. Aromatase cytochrome P450, product of *CYP19* gene, catalyzes the final rate limiting step in the biosynthesis of estrogens from androgens (Conley et al., 2001). In most mammals, expression of *CYP19* gene is regulated in tissue-specific manner by alternative use of different promoters (Sharma et al., 2009). It has been reported that the increased expression of aromatase mRNA from small to large follicles

* Corresponding Author : Dheer Singh. Tel: +91-184-2259135, Fax: +91-184-2250042, E-mail: drdheer.singh@gmail.com
[1] Animal Biochemistry Division, National Dairy Research Institute, Karnal- 132001, Haryana, India.
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indicates the high 17β-estradiol synthesis in antral follicles, an essential requirement for the follicular development and maturation in bovine (Evans et al., 2004) and buffalo ovary (Onteru et al., 2008). Thus, the *CYP19* gene is one of the crucial genes among the cluster of genes regulating the recruitment and selection of dominant follicle prior to ovulation. Though various primary cell culture system and granulosa cell line have been established and have enabled the study of *CYP19* gene regulation *in vitro*, but most of these primary cell culture system as well as cell lines have the limitation of maintaining the *CYP19* gene expression due to luteinization of granulosa cells which leads to down regulation of this gene in culture conditions containing serum. Accordingly, serum free culture conditions optimized in our laboratory have also been used for validating the RNAi experiments. As our laboratory is working on many aspects of *CYP19* gene (Onteru et al., 2008; Sharma et al., 2009; Ghai et al., 2010; Monga et al., 2011), which is a crucial reproductive gene, the gene was taken into consideration. The aim and basic idea of the present study was therefore to evaluate and validate the use of RNAi in cultured buffalo granulosa cells so that it can be used further as an effective tool for study of buffalo reproductive physiology as the work using this technique still lags behind in this species. In addition, efforts have also been made to compare the knockdown efficiency of siRNA duplex in serum and serum free granulosa cell culture.

## MATERIALS AND METHODS

### siRNA duplex and vector plasmids

The target sequences in the *CYP19* gene used for siRNA designing (acc. no. DQ407274.2) and the designed siRNA sequences are given in Table 1. The siRNA sequences were designed and synthesized by Qiagen, Australia and were annealed in RNase free conditions according to manufacturer's instructions. siRNA duplex targeted against the pGL3-luciferase vector and the control labeled siRNA duplex were purchased from Ambion, USA and Integrated DNA Technologies (IDT) respectively.

The plasmid encoding green fluorescent protein (pEGFP-N1) under the CMV promoter (Clontech, USA) was used for fluorescence reporter assay. The vector pEZ-b7SK-shEGFP expressing the shRNA against the EGFP gene was a kind gift by Dr. Timothy J Doran.

### Collection of ovaries

Buffalo ovaries (200 approx.) were collected from commercial abattoir, Delhi within 10-20 min after slaughtering in chilled normal saline (0.9% NaCl) containing 100 U/ml of penicillin and 100 μg/ml of streptomycin 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.

### Cell culture

Healthy, developing follicles were assessed by the presence of vascularized theca externa and clear amber follicular fluid with no debris. Follicles were dissected free of surrounding tissues using a sharp pair of scissors. The follicular fluid was aspirated from small (≤5 mm) and medium antral follicles (≤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 finally separated by centrifugation at low speed (1,500 rpm) for 4-6 min to pellet out the cells. The cells were washed in Dulbecco's Modified Eagle's Medium (DMEM) containing penicillin (100 U/ml) and streptomycin (100 μg/ml). Cell number and viability were estimated in haemocytometer using trypan blue exclusion method.

All the culture reagents were purchased from Sigma Aldrich unless otherwise stated. Both serum and serum free cultures were practiced. For culture system with serum, cells were seeded in 24 well tissue culture plates (Nunc, USA) at a density of $1\times10^5$ viable cells in 1 ml of DMEM with 3% Fetal Bovine Serum (Hyclone, USA), L-Glutamine (3 mM/ml), Penicillin (100 U/ml) and Streptomycin (100 μg/ml) and were maintained at 37°C and 5% $CO_2$ in air. After the first 24 h, culture medium was replaced with serum free media containing protease free BSA (0.1%), Selenium (4 ng/ml), Transferrin (2.5 μg/ml), Androstenedione (2 μM/ml), insulin (10 ng/ml), non-essential amino acid mix (1.1 mM/ml). Similar concentration of L-glutamine and antibiotics were used as stated above. The cells were then maintained continuously under serum free conditions. For serum free culture, cells were maintained under serum free conditions throughout beginning from the time of plating of the cells. For serum free culture, cells were plated at density of $1\times10^6$ viable cells in 1 ml of DMEM. The composition of serum free culture media used was the same as stated above and as described previously (Gutierrez et al., 1997). Culture medium was replaced after every 48 h. Cells were plated for

**Table 1.** siRNA sequences designed against *CYP19* gene

| Name | Target sequence (5′-3′) | Sense strand | Anti-sense strand |
|---|---|---|---|
| siRNA 1 | TAC AAC AAG ATG TAT GGA GAA | (5′CAA CAA GAU GUA UGG AGA A3′) dTdT | (5′UUC UCC AUA CAU CUU GUU G3′)dAdA |
| siRNA 2 | TTG CGC TGT GGT GAT GAT GAA | (5′GCG CUG UGG UGA UGA UGA A3′) dTdT | (5′UUC AUC AUC ACC ACA GCG C3′)dAdA |

92 h and 700 µl of media was replaced after every 48 h.

**Transfection of siRNA duplexes and vector plasmids**

Two different siRNA sequences were used. Different concentrations of siRNA i.e. zero, 50 nM and 100 nM were introduced in cultured cells. 50 nM each of the two siRNA were also co-transfected to analyze the additive effects of siRNA on gene expression. Scrambled siRNA (Santa Cruz, USA) was used as a negative control. The siRNA duplexes were diluted in DMEM. About 100 µl of medium was used for dilution per well. The medium used for transfection was both antibiotic and serum free. Lipofectamine™ 2000 reagent (Invitrogen, USA) was used as the transfection reagent. About 5 µl of lipofectamine™ 2000 per well was diluted in 100 µl DMEM lacking in serum and antibiotics and incubated at room temp for 5 min. Both the diluted solutions of siRNA and lipofectamine™ were then mixed gently by slow pipetting and kept at room temp in dark for 20-25 min to allow lipid-siRNA complex formation. During the time of this complex formation, medium from the culture plates was replaced with plain medium lacking serum and antibiotics thereby washing the cells to remove any serum contamination if remaining. Following complex formation, l ml of medium containing the lipid-siRNA complex was then added to each well in culture plates. Culture plates were maintained at 37°C and 5% $CO_2$ in air for 6 h. Following 6 h of transfection, culture plates were taken out and the transfection medium was removed and replaced with treatment medium The treatment medium was comprised of DMEM containing 25 ng/ml ovine Follicle stimulating hormone (FSH) and 50 ng/ml long R3 Insulin like Growth Factor-I (IGF-I) along with all the serum free media components stated as above. The treatment was given for a minimum of 24 h for analysis of effects of siRNA on *CYP19* gene expression and estradiol (hormone) production. The culture medium from each of the control and experimental groups was collected for estradiol concentration estimation using Enzyme immune assays. To verify the uptake of siRNA by the cultured granulosa cells, cells were transfected with control fluorescently labeled siRNA (siCY3) using the same methodology as above. The cells were washed thrice with Phosphate Buffer Saline (PBS) and were then suspended in PBS for fluorescence examination of the control labeled siRNA. For fluorescence microscopy, the cells were cultured on 8 well chamber slides with culture conditions same as above (volume of the culture medium was about 300 µl).

Briefly, for plasmid vector transfection (EGFP-N1), about 2 µg of plasmid DNA per well was used for transfection in 24 well culture plates containing 1 ml medium in each well. The other transfection parameters used were the same as for the siRNA transfection stated above. Culture wells transfected only with EGFP-N1 vector served as controls for the experiment. For the knockdown of luciferase reporter vector, siRNA directed against the pGL3-lucifearse mRNA was co-transfected along with the luciferase vector using the similar conditions of transfection as above. pGL3-control vector with SV40 promoter (a positive control of plasmid transfection) was used for the same and pRL-TK (Renilla-Thymidine Kinase) was used as an internal control. The activity of pGL3-control vector was normalized with that of pRL-TK. Three different concentrations of siRNA i.e. 10 nM, 20 nM and 50 nM were transfected along with the control vector. Following 6 h of transfection, the transfection medium was replaced with normal serum free medium. After 18-24 h, the cells were washed with PBS and lysed in Passive Lysis Buffer (PLB). About 25 µl of PLB was added to each well for lysis. The 10 × PLB provided in the Dual Luciferase kit (promega, USA) was diluted to 1 × PLB with nuclease free water.

**RNA isolation and reverse transcriptase PCR (RT-PCR)**

Total RNA was isolated using trizol (MRC). Isolated RNA was used immediately for RT-PCR or stored at -80°C until use. The RNA was quantified spectrophotometrically and RNA integrity was evaluated by denaturing agarose gel electrophoresis.

A total of 100 ng RNA was used for each of the set of experiments. cDNA synthesis was done using the First strand cDNA synthesis kit (Fermentas, Germany) using random hexamer primer. The reaction mixture contained 100 ng of total RNA, 1 µl of random hexamer (0.2 µg/µl) and DEPC treated water up 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 DTT), 1 µl of RNase inhibitor (20 IU), 2 µl of dNTP mix (10 mM), 2 µl of M-MuLV reverse transcriptase (200 IU) to a final volume of 20 µl. The contents were incubated at 25°C for 10 min, 42°C for 30 min and 95°C for 3 min. The prepared cDNA was then analyzed using real time PCR. *CYP19* gene specific primers were used for amplification (Table 2). The primers were

**Table 2.** Primer sequences

| Gene name | Sequence (5′-3′) | Acc. No. | Product size (bp) |
|---|---|---|---|
| *CYP19* | For<br>CCTGTGCGGGAAAGTACATCGC<br>Rev<br>TCTTCTCAACGCACCGATCTTG | DQ407274.2 | 105 |

designed from buffalo *CYP19* gene mRNA sequence using Primer 3 software.

**Real time PCR**

For quantitative PCR, cDNA prepared from 100 ng of RNA was amplified with LC 480 SYBR Green master mix (Roche) in a 12 µl reaction mixture. Amplification and quantification was performed using light cycler real time PCR (Roche Diagnostics, Switzerland) under the following cycling conditions: 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, 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 product was used to generate standard curve. For plasmid dilutions, range covering over eight orders of magnitude ($2.5\times10^{-15}$ to $2\times10^{-10}$ g DNA per reaction) were freshly diluted every time from the stock concentration of $10^{-9}$ g DNA/µl. Copy number was then calculated relative to the amount of RNA that was subjected to cDNA preparation. For negative control, PCR reaction with all the PCR reagents other than the added cDNA was used.

**Measurement of estradiol and progesterone concentration**

The concentration of estradiol was determined using Enzyme Linked Immunosorbent Assay (ELISA) (Omega diagnostics, UK). The estradiol and progesterone standard curves were generated from the standards provided in the kit which ranged from 0 pg/ml to 1 ng/ml and 0 ng/ml to 50 ng/ml, respectively. Estimation was performed as per the manufacturer's instructions using about 25 µl of spent medium for hormone estimation.

**Dual luciferase reporter assay**

In the DLR Assay, the activities of firefly (*Photinus pyralis*) and *Renilla* (*Renilla reniformis*, also known as sea pansy) luciferase were measured sequentially from a single sample using luminometer (Turner BioSystems, Luminescence Modulus single tube). Quantitation of luminescent signal from each of the luciferase reporter enzymes were performed immediately following lysate preparation as per the kit protocol instructions. The lysate was transferred into a tube or vial and stored at -70°C until ready for assay.

**Statistical analysis**

We performed statistical analysis using Prism Software (Graph Pad Software, Inc., San Diego, CA, USA). Both one-way and two-way analysis of variance (ANOVA) was used to analyze the data as shown with the respective figure legends. The difference between the means was compared using least significant difference (LSD) at $p<0.05$. All experimental data are presented as the mean±SEM of at least three (n = 3) independent experiments. The test of significance used and letters of significance have been described in the respective figure legends. The relationship between percentage of gene expression remaining and knockdown and *CYP19* gene and estradiol decline was evaluated using a linear correlation model and statistical regression analysis.

## RESULTS

Figure 1A and 1B shows the granulosa cells cultured under serum and serum free conditions at 96 h of culture respectively. As shown in Figure 1E, for the visual standardization of transfection of siRNA, cells transfected with control Cy3™ labeled siRNA duplex emitted red fluorescence showing the successful uptake of labeled siRNA by granulosa cells. The Cy3™ labeled transfection control duplex has an emission max of 570 nM and so the fluorescence was observed with a green filter covering the wavelength maxima of the siRNA using an inverted fluorescence microscope (Nikon Diaphot). Green fluorescence emission was visualized following transfection of cells with fluorescent vector, EGFP-N1 vector (Figure 1C). However, successive knockdown of fluorescence was observed after its co-transfection with vector (pEZ-b7SK-shEGFP) expressing siRNA against the EGFP gene (Figure 1D). This vector features the highly efficient bovine 7SK promoter which targets and suppresses the reporter gene encoding EGFP.

For the quantitative knockdown, cells co-transfected with pGL3-luciferase control vector, a positive control of plasmid transfection (with SV40 promoter) and siRNA targeting the luciferase vector mRNA showed significant knockdown in luminescence. Initially, cells transfected only with pGL3-luciferase vector showed very high luminescence. The luminescence from firefly luciferase was normalized with renilla luciferase (internal control). Normalizing the activity of the experimental reporter to the activity of the internal control minimized the experimental variability caused by differences in cell viability or transfection efficiency. Following its co-transfection with siRNA, significant knockdown in comparison to control was observed even the lowest concentration of 10 nM siRNA. However, 50 nM siRNA conc. was seen to have the highest degree of decline in luminescence (Figure 2).

As there is substantial evidence supporting the fact that serum is inhibitory to the maintainenece of *CYP19* gene expression, both serum free culture and culture medium along with serum were standardized for transfection.

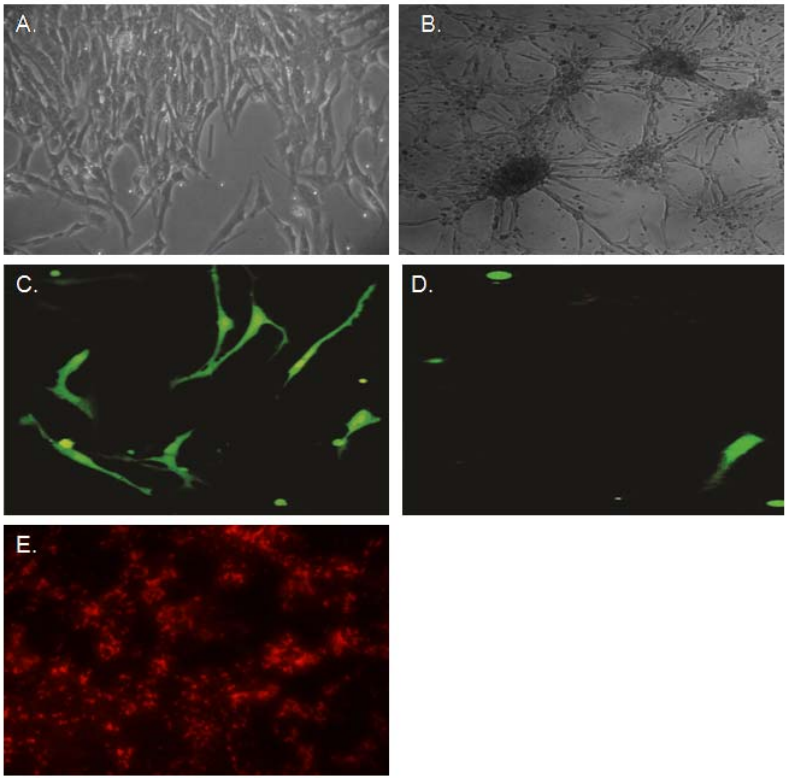


**Figure 1.** Transfection of buffalo granulosa cells with fluorescent plasmid vector, vector generating siRNA and siRNA duplex positive control. A) and B) buffalo granulosa cells cultured with serum and under serum free conditions at 96 h of culture respectively, C) fluorescence emission obtained after transfection of granulosa cells with EGFP-N1 vector, fluorescence measured at wavelength of 510 nM, D) knockdown of fluorescence emission achieved following co-transfection of EGFP-N1 vector along with the vector (pEZ-b7SK-shEGFP) generating siRNA, E) labeled siRNA duplex used as a positive control of siRNA duplex transfection.

Absolute quantification was used for quantifying the transcript abundance of the gene. Equal amount of RNA was utilized for cDNA preparation for different concentration of siRNA transfected. Gene copy number was then calculated and was in turn normalized to the initial amount of RNA used for cDNA preparation. For culture system with serum, siRNA (siRNA1) targeting the *CYP19* gene coding region was transfected. As shown in Figure 3A, significant decrease in copy no. of *CYP19* gene ($p \leq 0.05$) was found for 100 nM siRNA concentration. Bar graph

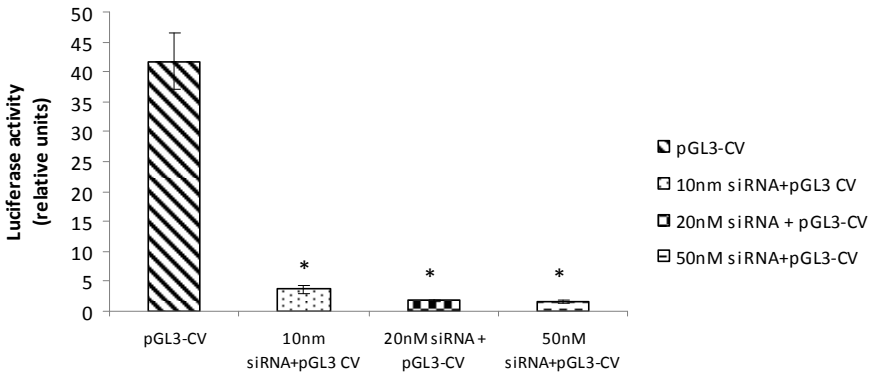


**Figure 2.** Co-transfection of granulosa cells with pGL3- luciferase control vector (pGL3-CV) - positive control of plasmid transfection along with the varying concentrations of siRNA duplex sequence targeted against the pGL3 - luciferase vector mRNA. Significant knockdown was achieved even with lowest concentrations of 10 nM siRNA. Renilla- Thymidine Kinase (pRL-TK) plasmid vector was used as an internal control. Results are expressed as the relative Luciferase activity, normalized to an internal control. Values in figures represent means±SEM of at least 3 different experiments. One way ANOVA was used for analysis. * $p<0.05$, significant differences from control condition (pGL3 control vector).

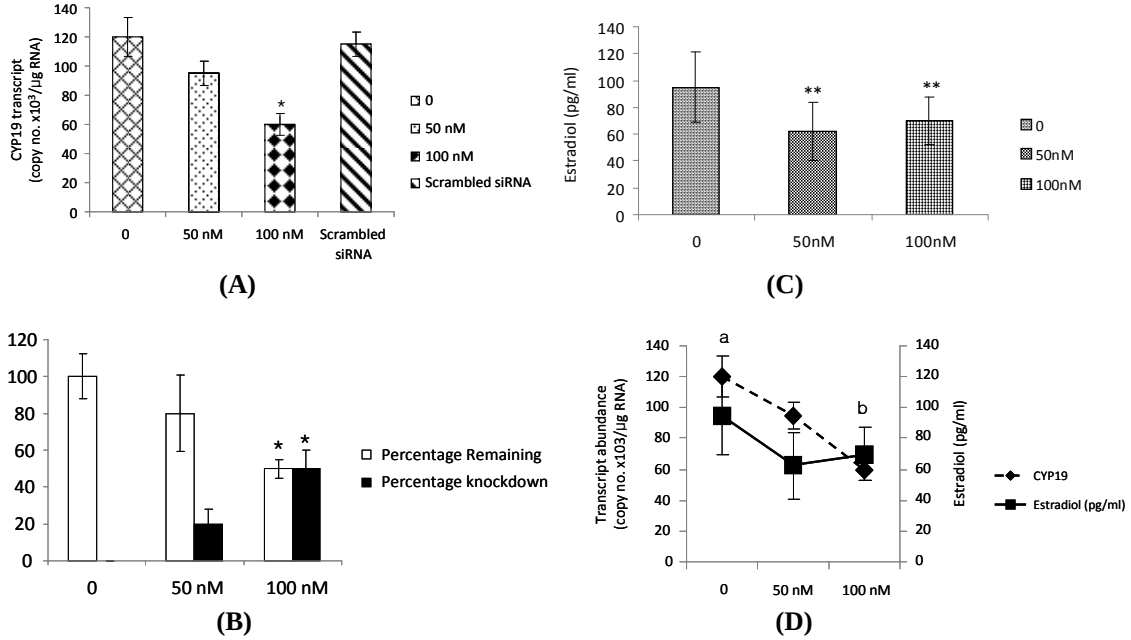


**Figure 3.** *CYP19* gene expression knockdown achieved following siRNA1 (directed against the *CYP19* gene coding region) transfection in culture system with serum. A) Absolute quantification of *CYP19* gene transcript represented as copies $\times 10^3$/µg RNA. Copy number is normalized relative to µg of RNA used initially for cDNA preparation, B) bar graph for the inverse relationship between percentage of gene expression remaining and percentage knockdown achieved, C) estimation of estradiol levels using ELISA, D) line graph showing how *CYP19* gene transcript abundance and estradiol content are related following siRNA transfection. Values in figures represent means ±SEM of at least 3 different experiments. For Figure 3A, 3B and 3C, one way ANOVA was used for analysis. * $p<0.05$, ** $p<0.01$, significant differences from control condition; for Figure 3D, two way ANOVA was used for analysis. The difference between the means was compared using least significant difference (LSD) at $p<0.05$. Figures with different letters differ significantly at $p<0.05$, * $p<0.05$. Regression analysis showed a non-linear relationship between percentage remaining and percentage knockdown (logarithmic $R^2 = 0.9$) whereas a linear relationship for *CYP19* gene and estradiol decline ($R^2 = 0.96$) .

depict that about 50 percent gene knockdown was achieved following transfection with 100 nM siRNA concentration (Figure 3B). Thereby, an inverse relationship was observed with increasing gene knockdown achieved following decrease in percentage of gene expression remaining after siRNA transfection. However, as shown in Figure 3C, hormone (17β-Estradiol) content for 100 nM siRNA concentration, although significantly lower than the control ($p\leq0.05$), a small increase (not significant) in comparison to 50 nM siRNA was observed. Figure 3D shows the linear relationship between *CYP19* gene transcript and 17β-Estradiol content, however, a slight increase in hormone content was seen for 100 nM siRNA. Scrambled siRNA was used as a negative control and no decrease in aromatase transcript concentration was observed following its transfection.

In the next experiment, serum free system was investigated for transfection of two different siRNA sequences. Figure 4 shows the analysis of siRNA transfection under similar culture conditions (serum free) and targeting different sequences of *CYP19* mRNA. For siRNA1 (targeting the coding region), significant copy number ($p\leq0.05$) was found to decrease for both the concentration of siRNA transfected. Co-transfection of the two different siRNA sequences targeting different sequences of *CYP19* gene showed a synergistic effect resulting in higher gene knockdown achieved in comparison to individual transfection of the two siRNA ($p\leq0.05$, Figure 4A). About 80 percent of gene knockdown was achieved when co-transfection of two siRNA was carried out whereas about 65 percent of gene knockdown was seen for 100 nM concentration of siRNA1 (Figure 4B). An intermediate concentration of 50 nM each of the two siRNA was chosen for co-transfection resulting in a significant gene knockdown. However, other concentration of the two siRNA, lower or higher for co-transfection can also be validated for transfection.

For the second siRNA transfected (siRNA2), as shown in Figure 4C, small decrease in *CYP19* gene transcript abundance was seen when transfected with 100nM siRNA (siRNA2) concentration, however, percentage gene knockdown achieved was only about 40 percent (Figure 4B). Therefore, percentage of gene expression remaining following siRNA transfection was higher for siRNA2 (Figure 4D). Thus concluding that siRNA1 is more efficient in suppressing the gene expression in comparison to

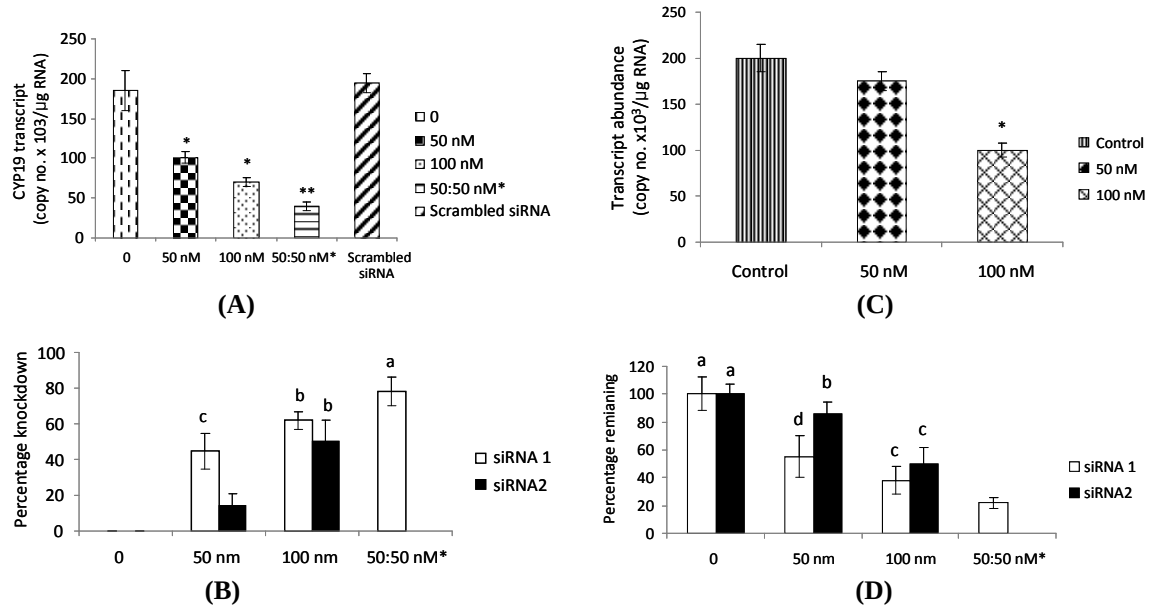


**Figure 4.** Transfection of serum free cultured buffalo granulosa cells with two different siRNA sequences (siRNA1 and 2). A) and C) transcript abundance of *CYP19* gene following transfection with siRNA 1 and 2 respectively, B) and D) bar graph showing the comparison of percentage of gene knockdown achieved and percentage expression remaining respectively when transfected with two different siRNA sequences targeted against different regions of *CYP19* gene. Values in figures represent means±SEM of at least 3 different experiments. For Figure 4A and 4C, one way ANOVA was used for analysis. * $p<0.05$, ** $p<0.01$, significant differences from control condition; for Figure 4B and 4D, two way ANOVA was used for analysis. The difference between the means was compared using least significant difference (LSD) at $p<0.05$. Figures with different letters differ significantly at $p<0.05$. 50:50 nM*- co-transfection of 50 nM each of 2 different siRNA sequences.

siRNA2. Comparison of the transcript abundance, percentage of gene expression remaining and knockdown achieved among the two different siRNA sequences, shows the higher potency of siRNA1 in causing the gene knockdown thereby reducing the transcript abundance of *CYP19* gene when compared with siRNA2. The siRNA1 targets the *CYP19* gene coding region while siRNA2 targets the UTR of transcript I.1 which is expressed only to a small extent (partially) in ovary. Significant knockdown in estradiol content was only achieved for 100 nM concentration of siRNA1 (Figure 5A, $p\leq0.05$) irrespective of siRNA2 (Figure 5B) or the co-transfection of the two siRNA for which no significant knockdown in the 17β-Estradiol content was achieved. A linear relationship was observed for the decline in *CYP19* gene expression and estradiol content for siRNA1 (Figure 5C) while the linearity between the decline in gene expression and hormone content for siRNA2 (Figure 5D) was demolished as no significant decline in hormone content was observed for any of the siRNA2 concentration.

Figure 6 shows the comparison of the transcript abundance and estradiol contents measured in the two different culture systems. Initially, transcript abundance of *CYP19* gene and hormone content (estradiol and progesterone) was compared at day 4 of culture with serum and under serum free conditions. Results showed that both gene transcript and estradiol contents (Figure 6A) significantly were higher under serum free conditions in comparison to serum containing cultures. However, progesterone content was seen to be higher under culture conditions with serum (Figure 6B). Also, significantly, higher *CYP19* gene copy number (Figure 6C) and 17β-estradiol content (Figure 6D) was achieved in serum free culture system ($p\leq0.05$) in comparison to culture system with serum both prior to siRNA transfection and also following the siRNA transfection. Consequently, validation of siRNA transfection for serum free culture system was accomplished and the system was thereby considered to be more reliant for the maintainenece of *CYP19* gene transcript than the serum culture. However, in context to morphology of the cells, no difference was seen among the cells transfected with siRNA and controls.

## DISCUSSION

In the present study, application of RNAi was investigated in cultured buffalo granulosa cells by both gene specific siRNA and reporter vector based small hairpin RNA (shRNA) gene knockdown. The study demonstrates that siRNA transfection is well optimized for gene specific knockdown and thus strongly suppressed the expression of *CYP19* gene and estradiol production in buffalo granulosa

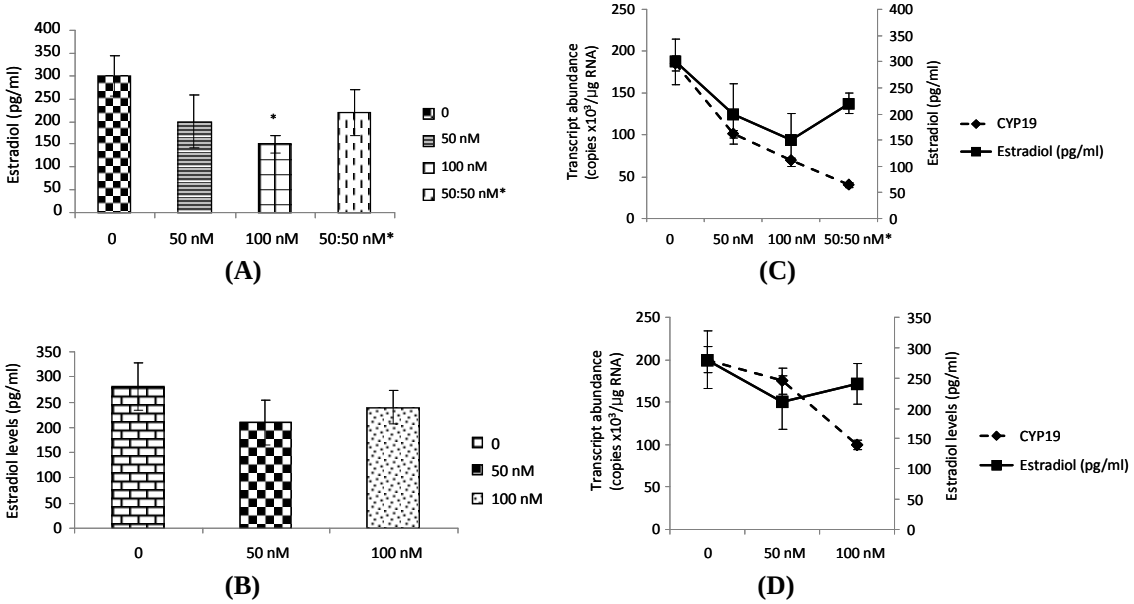


**Figure 5.** Estimation of estradiol in siRNA transfected cells using ELISA. A) and B) depicts the estradiol levels for the first (siRNA1) and second siRNA (siRNA2) transfected respectively in serum free culture conditions respectively. Significant knockdown in estradiol levels was only evident for 100 nM of siRNA1, C) and D) line graph relating *CYP19* gene transcript abundance and estradiol levels following transfection with siRNA1 and 2 respectively. A direct correlation between estradiol levels and *CYP19* gene expression was observed only for siRNA1. Values in figures represent means±SEM of at least 3 different experiments. For Figure 5A and 5B, one way ANOVA was used for analysis. * $p<0.05$, significant differences from control condition; for Figure 5C and 5D, two way ANOVA was used for analysis. The difference between the means was compared using least significant difference (LSD) at $p<0.05$. Figures with different letters differ significantly at $p<0.05$. 50:50 nM*- co-transfection of 50 nM each of 2 different siRNA sequences. For Figure 5C, regression analysis showed a linear relationship for *CYP19* gene and estradiol decline ($R^2 = 0.96$).

cells. This is the first report for validation of plasmid and siRNA transfection and *CYP19* gene knockdown in buffalo (*Bubalus bubalis*) granulosa cells. *CYP19* gene was used as a model to demonstrate the technique's validation. As far as our knowledge is concerned, this is the first serum free culture system developed for gene specific siRNA transfection with cells not being exposed to any concentrations of serum since the day of plating of the cells. The system was developed keeping in view the concept of luteinization of granulosa cells which leads to loss of aromatase mRNA expression in serum containing cultures and thus is difficult to maintain in culture systems containing serum.

The effects of suppression also vary according to the specificity of the target sequence. Certain studies have revealed that target site selection is of key importance in determining the potency and specificity of gene silencing (Holen et al., 2002; Kumar et al., 2003). Kobayashi et al. (2007) have demonstrated the use of gene specific siRNA for the knockdown of target gene in bovine cultured granulosa cells. In the present work, two different sequences of *CYP19* mRNA were selected for suppression; siRNA1 which was designed against the coding region of *CYP19* gene and siRNA2 was designed from the Untranslated Region (UTR) of exon I.1. Estimates of *CYP19* gene transcript concentration in different tissues revealed that the *CYP19* gene expression in granulosa cells is predominantly regulated by PII and to a minor extent by PI.1 (Sharma et al., 2009). PI.1 is almost exclusively responsible for *CYP19* gene expression in placenta and residual expression in corpus luteum. Thus siRNA2 designed against the UTR of exon I.1 was less potent in causing the transcript knockdown and reduction in hormone content in the granulosa cell culture derived from the preovulatory stage (small and medium follicles). Co-transfection of the two siRNA sequences proved to be still more effective which led to down regulation of both the transcript (II &I.1, residual expression of PI.1 in ovary) derived *CYP19* gene expression. It is possible that other sequences might be more effective in down regulating the gene at even lower concentration. Thus, target site selection and siRNA design have profound influence on the level and specificity of gene silencing.

For the initial validation of plasmid and siRNA transfection, culture containing serum was used while rest of the experiments on *CYP19* gene knockdown was done in serum free culture. Serum free culture system of buffalo granulosa cells was found to be more appropriate for the maintainenece of *CYP19* mRNA. Both serum and serum free culture system were validated for transfection. Many

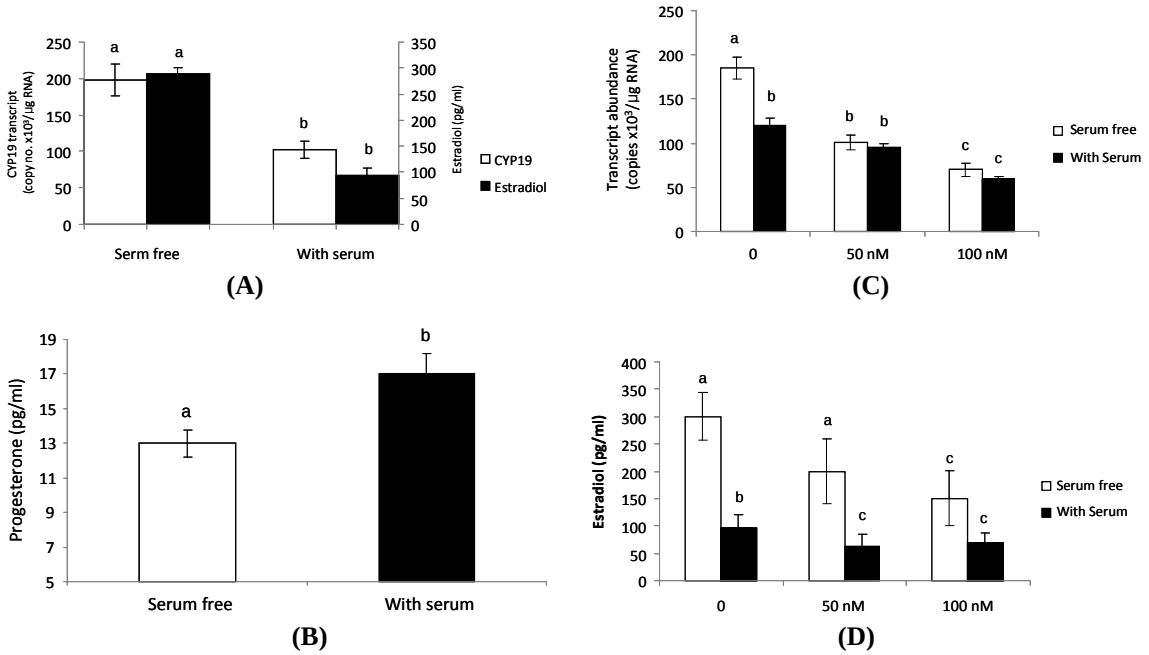


**Figure 6.** Comparison of serum and serum free culture systems for *CYP19* gene transcript abundance and Estradiol levels. A) depicts the comparison of *CYP19* gene copy number and estradiol concentration at day 4 of culture under serum containing and serum free conditions, B) comparison of progesterone contents in the two systems, C) copy number of *CYP19* gene transcript in the two culture systems with significantly lesser transcripts in culture system with serum, D) for estradiol levels again, significantly lesser hormone levels were maintained in culture system with serum. Values in figures represent means ±SEM of at least 3 different experiments. For Figure 6B, one way ANOVA was used for analysis. Figures with different letters differ significantly at $p<0.05$; for Figure 6A, 6C and 6D, two way ANOVA was used for analysis. The difference between the means was compared using least significant difference (LSD) at $p<0.05$. Figures with different letters differ significantly at $p<0.05$.

references from literature quote that serum has profound inhibitory effects on aromatase activity. Ackermann et al. (1994) have shown that aromatase expression increased with time in culture under serum free conditions in JEG-3 cells and therefore changing to serum free conditions after first 24 h was an essential requirement for the experiment. Thereby, it was stated by the above authors that serum uncontrollably and significantly affects aromatase expression. Also, in porcine granulosa cells, insulin and FSH could not prevent the loss of aromatase activity. It was assumed that some specific component is lacking in the medium that is required for maintaining the aromatase activity or perhaps a serum component is specifically inhibitory (May et al., 1981). In monolayer culture of human term placental cells, those cells deprived of serum showed significantly higher aromatase specific activity over the entire culture period compared with serum-grown cells (Lobo and Bellino, 1989). Thus in the present study, serum free culture system was optimized for transfection and was found to be more favorable for supporting the *CYP19* transcripts. Those cells deprived of serum showed higher *CYP19* gene transcript abundance. Absolute quantification was used to calculate the transcript abundance (Nimz et al., 2009). The percentage of gene knockdown achieved in serum free culture system was about 65 percent for siRNA1 and 80 percent after co-transfection of two different siRNA sequences when compared to 50 percent gene knockdown accomplished in culture system with serum. Also, cells were shown to exhibit increased estradiol contents suggesting an elevated aromatase activity in serum free culture system. Of the two different concentrations of siRNA introduced in the cultured granulosa cells, siRNA concentration of 100 nM was considered to be the effective dose both in interfering the gene expression and hormone production following treatment with FSH and growth factor like IGF1. Dose and time dependent studies for FSH and IGF1 have been standardized in our laboratory (Unpublished data). It has been discovered that the most important role of IGF1 appears to be reliant on its ability to synergize with gonadotropins and to amplify their steroidogenic output (Adashi et al., 1985; Veldhuis et al., 1986; Adashi et al., 1988; Urban et al., 1990; Balasubramanian et al., 1997). Maintainenece of *CYP19* gene transcript in serum free culture in itself is a task. Earlier studies in buffalo have reported that the inability to maintain aromatase activity in serum free culture conditions has been a major challenge in development of *in vitro* systems for culture of granulosa cells (Shanmugam et al., 2009). We in our laboratory have developed a serum free culture system of granulosa cells that well maintains

aromatase gene expression and activity (Unpublished data). The system was then optimized for siRNA and plasmid transfection. Though cows are very closely related species to buffaloes, the results from bovine granulosa cell culture are not directly translatable to buffaloes as there are several marked physiological differences between cow and buffaloes. The fertility in water buffalo (*Bubalus bubalis*) is much lower than that in cattle (*Bos taurus*). Poor breeding efficiency is attributed to late onset of puberty, seasonality, poor estrus expression, and long calving intervals (Drost, 2007). The ovaries of mature water buffalo (El-Wishy, 2007) are smaller than in the bovine. Also, Terzano, 2007 has described that physiologically, the buffalo ovary shows insufficient reproductive potentiality as the number of follicles (primary and antral) is less than those found in cattle. But still in buffalo, there is nearly complete lack of information on the factors controlling the selection of the dominant follicle, the period of functional dominance and the effects of environmental factors, such as climate and nutrition, on follicular dynamics (Manik et al., 2002).

In order to compare the two culture systems, only one siRNA sequence (siRNA1; against the coding region of *CYP19*) was transfected in serum containing culture which was found to cause significant gene knockdown in serum free culture. Serum free culture system was thereby validated for transfection of siRNA duplexes and was thus known to efficiently maintain the aromatase gene transcript abundance. For siRNA transfection control, fluorescently labeled siRNA (Cy3 siRNA) was used and red fluorescence elicited serves as a positive control for the successful uptake of the siRNA duplex.

The work demonstrated that a close association could not be observed between gene expression knockdown and the reduction in estradiol contents. Literature has evidences for the same. For instances, estradiol concentrations declined significantly before P450arom mRNA levels were known to decline (Xu et al., 1995). In rat gonandal tissues, dissociations between P450arom mRNA, protein levels and aromatase have been well demonstrated (Hickey et al., 1989; Brandt et al., 1990). Sourdaine et al. (1996) reported that there wasn't any significant association between P450arom mRNA and aromatase activity in human breast tumors. Thus a direct correlation between the mRNA levels and enzyme activity cannot be assumed. 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 estradiol from the extant enzyme remaining after the aromatase mRNA suppression. The hormone treatments selected during this initial phase was based on the study of Gutierrez et al. (1997) who justified the culture system to maintain granulosa cell phenotype. In the present study, the estradiol content was significantly higher with low progesterone content in serum free culture in comparison to serum containing cultures. However, quantitatively, *in vitro*, in both serum as well as serum free culture, the progesterone production was found to be higher than estradiol content. Similarly, all the serum free granulosa cell culture system used earlier in both bovine (Gutierrez et al., 1997; Silva and Price, 2000) and buffalo (Shanmugam et al., 2009) showed the similar pattern of estradiol and progesterone production.

Furthermore, both plasmid vector transfection and co-transfection were carried out. Transfection of plasmid EGFP-N1 expressing the green fluorescent protein was seen to give green fluorescence emission when observed under the fluorescence microscope at a wavelength of 510 nm. However, co-transfection of the plasmid EGFP-N1 along with the plasmid expressing the siRNA against the green fluorescent protein (pEZ-b7SK-shEGFP) resulted in the appropriate knockdown of the green fluorescence emission. Knockdown effect of siRNA molecules is basically transient due to the gradual reduction of cellular siRNA concentration by cell divisions, which sometimes leads to inefficient gene silencing. Thus, optimization of transfection conditions is essentially required for the sufficient introduction of siRNA molecules into the cells. As a result vector based siRNA expression system in which shRNA is expressed by RNA polymerase III promoters (U6, 7SK or H1) provides a stable gene silencing pattern in comparison to the chemically synthesized small interfering RNA (siRNA). The vector contains a replication origin for mammalian cells and can be maintained even after multiple cell divisions, enabling a stable knockdown. A number of vectors for expression of shRNA have thus now been developed. The work relating to the use of vector plasmid DNA to cause the gene specific knockdown has also been reported (Lambeth et al., 2006) and is being used in the present study. For the knockdown of green fluorescence emission, siRNA duplexes were co-transfected with GFP vector (Hirano et al., 2004).

## IMPLICATIONS

Results of the present study suggest that gene silencing using RNAi is well validated and is useful in analysis of specific gene function in buffalo granulosa cells. In addition, study also suggests that serum free culture conditions favors gene silencing effect of siRNA duplex as compared to culture conditions with serum. RNAi - a valuable research tool is another step forward to gene regulation by the interfering effects of siRNA, thereby enabling the elucidation of molecular mechanisms underlying reproductive disorders as well as in understanding the functional role of the gene of interest in mammalian

reproductive physiology.

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## REFERENCES

Ackermann, K., J. Fauss and W. Pyerin. 1994. Inhibition of cyclic AMP-triggered aromatase gene expression in human choriocarcinoma cells by antisense oligodeoxynucleotide. Can. Res. 54:4940-4946.

Adashi, E. Y., C. E. Resnick, A. M. Brodie, M. E. Svoboda and J. J. Van Wky. 1985. Somatomedin-C mediated potentiation of follicle stimulating hormone-induced aromatase activity of cultured rat granulosa cells. Endocrinology 117:2313-2320.

Adashi, E. Y., C. E. Resnick, E. R. Hernandez, J. V. May, M. Kecht, M. E. Svoboda and J. J. Van Wyk. 1988. Insulin-like growth factor-I as an amplifier of follicle stimulating hormone action: studies on mechanism(s) and site(s) of action in cultured rat granulosa cells. Endocrinology 122:1583-1591.

Aravin, A. A., M. S. Klenov, V. V. Vagin, Ya. M. Rozovskii and V. A. Gvozdev. 2002. Role of double-stranded RNA in eukaryotic gene silencing. Mol. Biol. 36(2):180-188.

Balasubramanian, K., H. A. Lavoie, J. C. Garmey, D. M. Stocco and J. D. Veldhius. 1997. Regulation of porcine granulosa cell steroidogenic acute regulatory protein (StAR) by IGF1: synergism with FSH or protein kinase A agonist. Endocrinology 138:433-439.

Brandt, M. E., D. Puett and S. J. Zimniski. 1990. Divergence between ovarian aromatase activity, estrogen, and androgen levels in the cycling rat. Endocrinology 126:72-79.

Brüning- Richardson, A. and G. A. McConkey. 2005. RNAi in the malaria parasite plasmodium. In: Gene Silencing by RNA Interference Technology and Application (Ed. M. Sohail).

Conley, A. and M. Hinshelwood. 2001. Mammalian aromatases. Reprod. 121:685-695.

Drost M. 2007. Bubaline versus bovine reproduction. Theriogenology 68:447-449.

EI-Wishy, A. B. 2007. The postpartum buffalo. I. Endocrinological changes and uterine involution. Theriogenology 97:201-215.

Evans, A. C. O., J. L. H. Ireland, M. E. Winn, P. Lonergan, G. W. Smith, P. M. Coussens and J. J. Ireland. 2004. Identification of genes involved in apoptosis and dominant follicle development during follicular waves in cattle. Biol. Reprod. 70:1475-1484.

Fire, A. and M. Nirenberg. 2005. RNA interference technology: From basic science to drug development. Cambridge University Press, Cape Town.

Ghai, S., R. Monga, T. K. Mohanty, M. S. Chuahan and D. Singh. 2010. Tissue-specific promoter methylation coincides with Cyp19 gene expression in buffalo (*Bubalus bubalis*) placenta of different stages of gestation. Gen. Comp. Endocrinol. 169: 182-189.

Gonzalez, G., L. Pfannes, R. Brazas and R. Straiker. 2007. Selection of an optimal transfection reagent and comparison to electroporation for the delivery of viral RNA. J. Virol. Methods 145(1):14-21.

Guerra-Crespo, M., J. L. Charli, V. H. Ia, G. Pedraza-Alva and Lerez-Martinez. 2003. Polyethylenimine improves the transfection efficiency of primary cultures of post-mitotic rat fetal hypothalamic neurons. J. Neurosci. Methods 127:179-192.

Gutierrez, C. G., B. K. Campbell and R. Webb. 1997. Development of a long-term bovine granulosa cell culture system: Induction and maintenance of estradiol production, response to follicle stimulating hormone, and morphological characteristics. Biol. Reprod. 56:608-616.

Hickey, G. J., R. B. Oonk, P. F. Hall and J. S. Richards. 1989. Aromatase cytochrome P450 and cholesterol side-chain cleavage cytochrome P450 in corpora lutea of pregnant rats: diverse regulation of peptide and steroid hormones. Endocrinology 125:1673-1682.

Hirano, T., N. Yamauchi, F. Sato, T. Soh and M. Hattori. 2004. Evaluation of RNA interference in developing porcine granulosa cells using fluorescence reporter genes. Reprod. Dev. 50:599-603.

Holen, T., M. Amarzguioui, M. T. Wiiger, E. Babaie and H. Prydz. 2002. Positional effects of short interfering RNAs targeting the human coagulation trigger tissue factor. Nucleic Acids Res. 30:1757-1766.

Khatri, K., A. Rawat, S. Mahor, P. N. Gupta and S. P. Vyas. 2006. Double stranded RNAs as gene silencing tool: An overview. Indian J. Biotech. 5:460-470.

Kobayashi, S., M. Sakatani, S. Kobayashi, K. Okuda and M. Takahashi. 2007. Gene silencing of cyclooxygenase-2 mRNA by RNA interference in bovine Cumulus-Granulosa cells. J. Reprod. Dev. 53:1305-1311.

Kumar, R., D. S. Conklin and V. Mittal. 2003. High-throughput selection of effective RNAi probes for gene silencing. Genome Res. 13:2333-2340.

Lambeth, L. S., T. G. Wise, M. S. Moore and T. J. Doran. 2006. Comparison of bovine RNA polymerase III promoters for short hairpin RNA expression. Anim. Genet. 37:369-372.

Lobo, J. O. and F. L. Bellino. 1989. Estrogen synthetase (Aromatase) activity in primary culture of human term placental cells: Effects of cell preparation, growth medium, and serum on adenosine 3', 5'-Monophosphate response. J. Clin. Endocrinol. Metabol. 69:868-874.

Manik, R. S., P. Palta, S. K. Singla and V. Sharma. 2002. Folliculogenesis in buffalo (*Bubalus bubalis*): a review. Reprod. Fertil. Dev. 14(5):315-325.

May, J. M. and D. W. Schomberg. 1981. Granulosa cell differentiation *in vitro*: Effect of insulin on growth and functional integrity. Biol. Reprod. 25:421-431.

Monga, R., S. Ghai, T. K. Datta and D. Singh. 2011. Tissue-specific promoter methylation and histone modification regulate CYP19 gene expression during folliculogenesis and luteinization in buffalo ovary, (Article in press).

Nimz, M., M. Spitschak, F. Schneider, R. Fürbass and J. Vanselow. 2009. Down- regulation of genes encoding steroidogenic

enzymes and hormone receptors in late preovulatory follicles of the cow coincides with an accumulation of intrafollicular steroids. Domest. Anim. Endocrinol. 37(1):45-54.

Onteru, S. K., D. Sharma, D. Singh and M. K. Sharma. 2008. CYP19 (cytochrome P450 aromatase) gene polymorphism in murrah buffalo heifers of different fertility performance. Res. Vet. Sci. 87:427-437.

Shanmugam, M., S. Pandita and P. Palta. 2009. Effects of FSH and LH on steroid production by buffalo (*Bubalus bubalis*) granulosa cells cultured *in vitro* under serum free conditions. Reprod. Domest. Anim. 45:922-926.

Sharma, D., S. Ghai and D. Singh. 2009. Different promoter usage for CYP19 gene expression in buffalo ovary and placenta. Gen. Comp. Endocrinol. 162:319-328.

Silva, J. M. and C. A. Price. 2000. Effect of follicle-stimulating hormone on steroid secretion and messenger ribonucleic acids encoding cytochromes P450 aromatase and cholesterol side-chain cleavage in bovine granulosa cells *in vitro*. Biol. Reprod. 62:186-191.

Sourdaine, P., P. Mullen, R. White, J. Telford, M. G. Parker and W. R. Miller. 1996. Aromatase activity and CYP19 gene expression in breast cancers. J. Steroid Biochem. Mol. Biol. 59:191-198.

Tezano, G. M. 2005. Follicular dynamics and reproductive technologies in buffalo. In: Buffalo production and Research. Rome: REU technical Series 67. pp. 109-136.

Urban, R. J., J. C. Garmey, M. A. Shupnik and J. D. Veldhuis. 1990. Insulin-like growth factor type-I increase concentrations of mRNA encoding CYP450scc enzyme in primary cultures of porcine granulosa cells. Endocrinology 127:2481-2488.

Veldhuis, J. D., R. J. Rodges, A. Dee and E. R. Simpson. 1986. The insulin-like growth factor, somatomedin C, induces the synthesis of cholesterol side-chain cleavage cytochrome P450 and adrenodoxin in ovarian cells. J. Biol. Chem. 261:2499-2502.

Xu, Z. Z., H. A. Garverick, G. W. Smith, M. F. Smith, S. A. Hamilton and R. S. Youngquist. 1995. Expression of messenger ribonucleic acid encoding cytochrome P450 side-chain cleavage, cytochrome P450 17a-hydroxylase,and cytochrome P450 aromatase in bovine follicles during the first follicular wave. Endocrinology 136:981-989.