Université de Montréal

PURIFICATION AND IDENTIFICATION OF SPECIFIC RNA-BINDING PROTEIN THAT BINDS TO THE 3'UTR REGION OF CYTOCHROME P450AROMATASE mRNA IN BOVINE GRANULOSA CELLS

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Mémoire présenté à la Faculté des études supérieures et postdoctorales
en vue de l'obtention du grade de
Maître ès sciences (M.Sc.)
en sciences vétérinaires
option reproduction

Juillet 2008

Université de Montréal

Faculté des études supérieures et postdoctorales

Ce mémoire intitulé

PURIFICATION AND IDENTIFICATION OF SPECIFIC RNA-BINDING PROTEIN THAT BINDS TO THE 3'UTR REGION OF CYTOCHROME P450AROMATASE mRNA IN BOVINE GRANULOSA CELLS

présenté par

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a été évalué par un jury composé des personnes suivantes

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ABSTRACT

Estradiol is important for follicle development and ovulation, as P450aromatase (P450arom) knockout mice do not ovulate. The mRNA encoding the bovine aromatase gene has a long (3.5kb) 3'-untranslated region (3'UTR) and a short half life (3-7h), suggesting that posttranscriptional regulation may be important for control of enzyme activity. UV crosslinking experiments showed that a small fragment (1kb), which lies between +592 and 1714 of the 3'UTR, bound to 2 proteins of approximately 70kDa and 97kDa respectively that are present in granulosa cells, but not in corpus luteum, lung, liver, kidney, pancreas or bladder extracts. The major objective was to isolate the 3'UTR binding protein(s) from granulosa cells extracts.

Multiple purification methods were used, including enriching RNA binding proteins by heparin affinity chromatography, followed by direct and indirect capture on solid-phase oligonucleotide bait. Purified proteins and RNA-protein complexes were analysed by LC/LC-MS; unfortunately, no candidate protein could be identified.

Key words: Estradiol, aromatase, Cyp19, 3'UTR, RNA-binding protein(s).

RÉSUMÉ

L'hormone stéroidienne estradiol est très important pour les processus de croissance folliculaire et de l'ovulation. Le souris null pour l'enzyme estradiol synthase (P450aromatase) est infertil; les follicules n'arrivent pas à ovuler. L'ARNm codant pour le gene P450aromatase possède une région non-traduite 3' ('3'UTR) très longue (3.5 kb), et a une courte demi-vie d'environ 3 à 7 h. Ceci nous suggère que l'activité de ce gène est sous comtrole post-transcriptionnel. Des études de fixation aux UV nous avon permit de conclure que l'UTR de cet ARN se lie à (ou aux) de(s) protéine(s) de poids moléculaire de 70 kDa qui se retrouve dans les cellules de granulosa mais pas dans d'autres tissus tels que le corps jaune, la vessie, les poumons, la rate et le pancréas. L'objectif de cette étude était d'isoler cette protéine de'extrait de cellule de granulosa.

Plusieures approches ont été utilisées, basé sur la chromatographie par affinité. Un extrait enrichi a été preparé par absorption de protéines de liaison à l'ARN sur les billes de héparine-agarose. La matrice d'affinité était le séquence d'ARN de l'UTR, qui a été attaché à un support solid de différentes façons, est les fractions protéiniques ont été éluées. Malheureusement, aucune fraction n'était suffissament pure pour permettre une identification par LC/LC-MS.

Mots-clés : Estradiol, aromatase, 3'UTR, Cyp19, protéine(s) de liaison à l'ARN.

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LIST OF ABBREVIATIONS

%	Percent
µg	Microgram
µl	Microlitre
℃	Degrees Celcius
ACTH	Adrenocorticotropic hormone
AC	Adenylyl cyclases
AR	Androgen receptor
ARE	AU-rich elements
ATP	Adenosine triphosphate
ATRISt	AA-related thioesterase involved in steroidogenesis
BMP	Bone morphogenetic proteins
Bp	Base pair
C*n*	Carbon atoms
C17	Estrogens
C19	Androgens
C21	Progestins
C27	Cholesterol
cAMP	Cyclic adenosine 3',5'monophosphate
CYP19	Aromatase gene
cDNA	Complementary deoxyribonucleic acic
CL	Corpus luteum
COX	Cyclooxygenase
cpm	Cost per mille
CTP	Cytidine triphosphate
DTT	Dithiothreitol

DF	Dominant follicle
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EMSA	Electrophoretic mobility shift assay
EPOX	Epoxygenase
ER	Estrogen receptor
ER α	Estrogen receptor alpha
ER β	Estrogen receptor beta
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
FIG- α	Factors in the germline alpha
FSH	Follicle stimulating hormone
FSHr	Follicle stimulating hormone receptor
G	G proteins
GC	Granulosa cell
GDF-9	Growth differentiation factor-9
GTP	Guanosine triphosphate
h	Hour
HDL	High density lipoproteins
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor binding protein
Kb	Kilobase
kDa	Kilodalton
LC-MS	Liquid chromatography-mass spectrometry
LDL	Low density lipoproteins
LH	Luteinizing hormone
LHr	Luteinizing hormone receptor

LPOX	Lipoxygenase
mM	Millimolar
M	Mole
mRNA	Messager ribonucleic acid
MW	Molecular weight
NADPH	Nicotinamide adenine dinucleotide phosphate
ORF	Open reading frame
P450arom	Cytochrome P450 aromatase
P450scc	Cytochrome P450 side chain cleavage
$P450_{17\text{-}OH}$	Cytochrome P450 17 hydroxylase
P2	Promoter 2
pH	Relative hydrogen proton (H+) concentration
PI3K	Phosphatidylinositol 3-kinase
PIs	Isoelectric points
PKA	Protein kinase A
PTEN	Phosphatase and tensin homolog deleted on chromosome 10
PLA2	Phospholipase A2
PMSF	Phenylmethanesulphonylfluoride
POF	Premature ovarian failure
PR	Progesterone receptor
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase-polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SF1	Steroidogenic factor one
Sox9	SRY-type high mobility group box 9
StAR	Steroidogenic acute regulatory protein
TGF	Transforming growth factor

TGF α	Transforming growth factor alpha
TGF β	Transforming growth factor alpha beta
UTP	Uridine triphosphate
UV	Ultraviolet rays
V	Volt
2 D	Two dimension
3 β -HSD	Three beta-hydroxysteroid dehydrogenase
7 β -HSD	Seven alpha-hydroxysteroid dehydrogenase
3'-UTR	Three prime untranslated region
5'-UTR	Five prime untranslated region

ACKNOWLEDGEMENTS

I have greatly profited and appreciated help from my supervisor, Dr. Christopher A. Price, over the last there years. Without his help, thesis would never have been done.

I would like to express my cordial gratitude to Dr. Bruce D. Murphy, Dr. Alan K. Goff, Dr. Derek Boerboom for their valuable comments and suggestions, and kindly help during my studies in Faculté de Médecine Vétérinaire.

I also want to appreciate all members of CRRA for their help.

To my Chinese friends in Canada, I want to thank all of you, and will miss the every moment we have been shared.

To my dear parents, thanks for your unreserved believing and encouraged me to keep going, when I faced perplexities and difficulties alone in Canada.

To my families and friends in China

INTRODUCTION

Growth and regression of bovine ovarian follicles occurs in 'waves', during which a single potentially ovulatory follicle predominates in the ovary (Fortune, Rivera et al. 2001; Ginther, Beg et al. 2001). One of the major characteristics of growing and potentially ovulatory follicles is the higher estradiol content within the follicle compared to regressing and atretic follicles. In cattle, estradiol is synthesized in granulosa cells from thecal androgens by the enzyme cytochrome P450 aromatase (P450arom) (Vanselow, Furbass et al. 2001). Estradiol is important for follicle development and ovulation, as P450arom knock-out mice do not ovulate (Fisher, Graves et al. 1998). The local actions of estradiol include induction of LH receptors on granulosa cells (Rosenfeld, Wagner et al. 2001), and it has been shown that estradiol may be necessary for follicle growth in cattle in vivo (Beg, Meira et al. 2003).

Levels of mRNA encoding P450arom are upregulated in rodent, human and bovine granulosa cells by FSH (Steinkampf, Mendelson et al. 1987; Fitzpatrick and Richards 1991; Manuel Silva and Price 2000; Garverick, Baxter et al. 2002) and insulin/insulin-like growth factor-1 (IGF1) (Steinkampf, Mendelson et al. 1988; Silva and Price 2002). In contrast, LH has been shown to downregulate P450arom mRNA levels in rats and cattle (Voss and Fortune 1993; Fitzpatrick, Carlone et al. 1997; Komar, Berndtson et al. 2001). The half-life of P450arom mRNA in bovine, rabbit and rat granulosa cells is between 3 and 7 hours (Fitzpatrick, Carlone et al. 1997; Hanoux, Bouraima et al. 2003; Sahmi, Nicola et al. 2006), whereas it is 12 h in a human granulosa cell line (Mu, Yanase et al.

2001). The translation inhibitor cycloheximide stabilized P450arom mRNA in rat (Fitzpatrick, Carlone et al. 1997) and bovine (Sahmi, Nicola et al. 2006) granulosa cells, insulin-stimulated P450arom mRNA is more stable than that stimulated by FSH (Sahmi, Nicola et al. 2006).

Although the regulation of mRNA stability is complex, the 3'-untranslated region (3'UTR) of many mRNAs plays an important role in mRNA degradation (Guhaniyogi and Brewer 2001) (Bakheet, Frevel et al. 2001)). AU-rich sequences within the 3'UTR are the main determinants of mRNA stability, particularly the AUUUA pentamer and UUAUUU(U/A)(U/A)U nonamer (Guhaniyogi and Brewer 2001) (Bakheet, Frevel et al. 2001). AU-rich elements (ARE) interact with specific RNA binding proteins in *trans* to stabilize or destabilize mRNA.

A RNA binding protein(s) was detected bound to 3'UTR of P450arom mRNA (Sahmi, previous studies), therefore we performed a number of studies to purify the putative Cyp19 3'UTR binding protein(s).

LITERATURE REVIEW

THE BOVINE OVARY

The ovary is one of the female reproductive organs, composed of cortex and medulla, and surrounded by the germinal epithelium. The cortex mainly consists of developing follicles, the corpus luteum (CL) and corpus albicans. The medulla consists of nervous, fibroelastic tissue and blood vessels. The structure of the ovary is illustrated in Figure 1.

The shape and size of the ovary vary both with species and the stage of estrous cycle. In cattle and sheep, the ovary is almond-shaped, whereas it is bean-shaped in horses. The estrous cycle of the cow is generally about 21 days, ranging from 17 to 24 days. Small follicles (1 to 3 mm) increase to larger follicles between days 1 - 18. Most large follicles (around 10 mm) persist between day 3 - 13, and after day 13 most large follicles will be replaced by new growing follicles with formation of the preovulatory follicle after day 18 (Spicer and Echternkamp 1986). The CL develops after ovulation. In the cow, the CL increases rapidly both in size and functions between days 3 - 16, and starts to regress after day 16. The CL is almost non-functional at days 18-19.

The ovary performs both exocrine (egg release) and endocrine (steroidogenesis) functions.

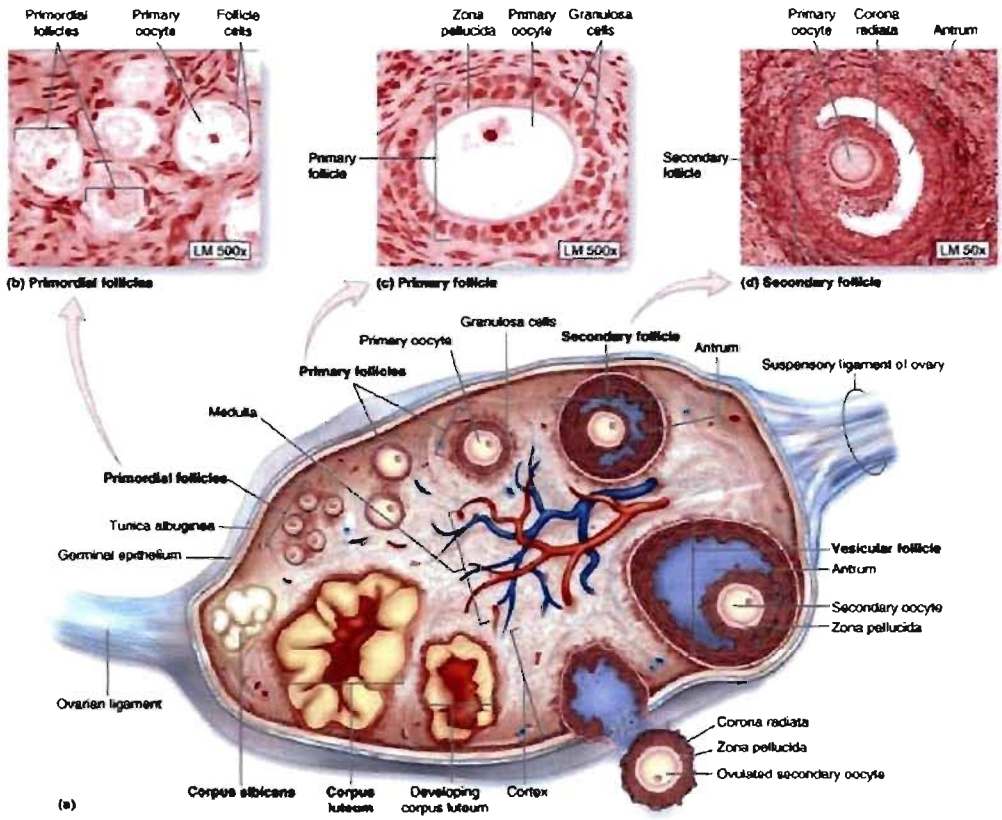

Figure 1: structure of the ovary and the formation of follicles

The ovary is composed of a cortex and a medulla. Diagram represents the various stages of developing follicles and corpus luteum.

(From http://academic.kellogg.cc.mi.us/herbrandsonc/bio201...)

FOLLICULOGENESIS

Follicles are the basic functional units of the ovary. Follicular development begins when follicles leave the pool of resting follicles and begin a process of growth, proliferation, and cellular differentiation that culminates in ovulation. The processes of follicular development have been known to be controlled by both extra-ovarian factors such as gonadotropic hormones from the pituitary gland, and intra-ovarian factors (Webb, Campbell et al. 1999). These factors determine whether follicles continue to develop or be diverted into atretic pathways.

Follicular growth and the regulation of follicular development

Follicular development is the physiological process during which a follicle develops from primordial stage through primary, secondary and tertiary stages.

The primordial follicle is composed of a primary oocyte and an outer single squamous layer of pregranulosa cells. Primordial follicles constitute the population of resting, non-growing follicles which form the source of growing follicles during the reproductive lifespan, and the number of primordial follicles declines progressively with age. Primordial follicles progress to atresia or entry into the growth phase. Once primordial follicles enter the growth phase, the growing follicle is characterized by the proliferation of granulosa cells (GCs) and by an increase in the size of the oocyte. The primary follicle is characterized by conversion of the flattened pregranulosa cells surrounding the oocyte into a single layer of cuboidal granulosa cells. A zona pellucida is formed around the oocyte. The secondary follicle is characterized by the proliferation of granulosa cells, such

that the oocyte is surrounded by several layers of granulosa cells. Primordial, primary and secondary follicles are collectively referred to as preantral follicles.

Tertiary and preovulatory follicles are classed as antral follicles. These follicles are characterized by the formation of a fluid-filled antrum, a well differentiated theca layer consisting of a theca interna and a theca externa, and differentiation of granulosa cells into cumulus granulosa cells which are ovulated with the oocyte, antral granulosa cells (closest to the antral cavity) and mural granulosa cells (closest to the basement membrane of the follicle). The formation of follicles is illustrated in Figure 1.

Follicular development is a dynamic process that can be divided into different separate stages: recruitment of primordial follicles from the resting pool into a growing pool; the continued growth from primary to small antral stage; selection of a single (in cattle) dominant follicle for rapid development while the growth of subordinate follicles is suppressed; and ovulation and luteinisation (Armstrong and Webb 1997).

In a number of species, primordial follicles undergo initial recruitment to leave the resting pool and enter the growing pool of primary follicles. Resting follicles are likely to be under inhibitory influences to remain dormant, therefore a decrease of these inhibitory influences and/or an increase of stimulatory factors allow the initiation of follicle growth (McGee and Hsueh 2000). The molecular signals which initiate the entry of a primordial follicle to the primary stage and beyond are uncertain, but multiple stimulatory and inhibitory factors are involved. PTEN (phosphatase and tensin homolog deleted on chromosome 10) is a major negative regulator of PI3K (phosphatidylinositol 3-kinase), and in mice lacking PTEN in the oocyte, the entire primordial follicle pool becomes

activated, leading to follicle depletion in early adulthood and premature ovarian failure (POF) (Reddy, Liu et al. 2008). The intra-oocyte PTEN-PI3K signaling pathway plays an important role in follicle activation through control of initiation of oocyte growth (Reddy, Liu et al. 2008). Other oocyte-secreted factors include GDF-9 (growth differentiation factor-9), BMPs (bone morphogenetic proteins), FIG-α (factors in the germline alpha) and c-kit receptor(Webb and Campbell 2007). In sheep, oocytes in primordial follicles express mRNA and proteins for c-kit, oestradiol receptor beta (ERβ) and BMP and transforming growth factor beta (TGFβ) receptors and binding proteins (McNatty, Reader et al. 2007). Kit-ligand and GDP-9 are highly expressed in secondary follicles. In cattle, preantral follicles express mRNAs encoding both IGFBP-2, -3 and type 1 IGF receptor (Armstrong, Baxter et al. 2002), and epidermal growth factor (EGF) also stimulates preantral follicle growth (Gutierrez, Ralph et al. 2000). It is generally accepted that gonadotropins are not involved in the initiation of follicle growth from the primordial follicle pool (Webb and Campbell 2007). Hypophysectomy does not inhibit this phase of growth (Dufour, Cahill et al. 1979), however, follicle stimulating hormone receptor (FSHr) is detected in bovine follicles with only one or two layers of granulosa cells (Bao and Garverick 1998), and FSH can accelerate the rate of preantral follicle development both in vivo (Campbell, Telfer et al. 2000) and vitro (Gutierrez, Ralph et al. 2000). Luteinizing hormone receptor (LHr) mRNA is first detected when the theca forms around the granulosa cells (Bao and Garverick 1998), presumably stimulating androgen precursor production by steroidogenic enzymes. During preantral growth, FSH and LH receptors coupled to the cyclic AMP second messenger system develop in granulosa and thecal cells respectively (McNatty,

Reader et al. 2007)

At the antral stage, most follicles undergo atresia and only a few survive to reach the preovulatory stage. Cyclic recruitment is characterised by transient increases in circulating FSH (McGee and Hsueh 2000). After a cohort of antral follicles escapes atresia (apoptosis), a species-specific number of follicles are selected for dominance. Dominant follicles (DF) grow faster than the rest of the cohort (subordinate follicles, SF) and produce higher levels of estrogens and inhibins. DF are likely to be more sensitive to FSH (Fauser and Van Heusden 1997), perhaps because of enhanced FSH and/or LH receptor expression or increased production of local growth factors (Xu, Garverick et al. 1995; Bao, Garverick et al. 1997; Evans and Fortune 1997). Local factors such as IGF-1 and TGF-α are also survival factors for preovulatory follicles (Chun, Billig et al. 1994). Antral follicle development occurs from a diameter of ~0.2-0.4 mm (size of antrum formation) to 2-4 mm in hypophysectomised sheep, and this follicle growth phase is commonly referred to as the gonadotropin–responsive phase as FSH accelerates the rate at which these follicles grow, but is not an essential requirement (Webb, Campbell et al. 1999). In contrast, follicle growth beyond 4 mm in diameter is gonadotropin dependent (Campbell, Scaramuzzi et al. 1995). Changes in the expression of mRNA for FSHr, LHr and steroidogenic enzymes are also demonstrated to occur at this stage of development (Webb and Campbell 2007). Some growth factor(s), such as GDF-9, BMP15 and perhaps other factors are thought to regulate gene expression in cumulus cells to enhance oocyte development, and in mural granulosa cells to regulate their response to pituitary hormones during antral follicle development (McNatty, Reader et al. 2007).

Follicular development in cattle

Growth and regression of bovine follicles occurs in waves. Most oestrous cycles consist two or three waves in cattle (Evans and Fortune 1997). Each wave of follicular growth is characterized by recruitment of a cohort of follicles, and only single follicle is selected for continued growth and becomes dominant. Luteolysis determine the fate of dominant follicles; if luteolysis occurs, the dominant follicle will ovulate, otherwise it undergoes atresia.

The first transient FSH rise occurs within 1 or 2 days of ovulation, which stimulates up to 24 small antral (a cohort) follicles to grow beyond 4mm in diameter (emergence; day 1-2 of the estrous cycle) (Ginther, Kot et al. 1997). As FSH concentrations begin to decline (on day 2-3 of the estrous cycle), one follicle is selected to continue to grow and become the dominant follicle, other follicles from the original cohort undergo atresia via apoptosis (Austin, Mihm et al. 2001). During this period, the dominant follicle grows (from 8.5mm at the end of selection to 12-20 mm) and produces high concentrations of estradiol, thereby maintaining low FSH levels to prevent any other cohort growth (Ginther, Bergfelt et al. 1999; Ginther, Bergfelt et al. 2000). Estradiol produced from the developing dominant follicle is involved in the negative feedback regulation of FSH. In an autocrine-paracrine mode, increasing estradiol production by the dominant follicle confers its ability to survive the decline in FSH, and results in expression of LHr on granulosa cells (Kolibianakis, Papanikolaou et al. 2005). Estradiol secretion by the first dominant follicle initial declines on day 6 and the follicle loses dominance between days 7 and 9, at which time another transient FSH rise occurs and stimulates emergence of a second

follicle wave (Sunderland, Crowe et al. 1994). During the dominance period of the second dominant follicle, if luteolysis occurs, a high frequency LH pulse pattern supports final differentiation of the second dominant follicle and prepares it for ovulation. If the corpus luteum is still actively suppressing LH pulse frequency, the second dominant follicle will also undergo atresia, and a third wave emerges giving rise to the third dominant follicle (Mihm, Crowe et al. 2002). The gonadotropin regulation of dominant follicle turnover is illustrated in Figure 2.

During the process of development of the dominant follicle in cattle, the shift from FSH- to LH-dependence was viewed as a pivotal event. In granulosa cells, there is an acquisition of LH receptors which may play a key role in the establishment and maintenance of follicular dominance, whereas FSH receptors during this process may only play a permissive role. LH controls the fate of the dominant follicle, high LH during the follicular phase leads to ovulation but sustained low LH during the luteal phase leads to regression and atresia (Valdez, Cuneo et al. 2005).

TGF β superfamily and IGF system

Dominant follicle selection and subordinate follicle regression are driven in part by their reliance from FSH to LH support. Other growth factors, such as the TGF-β (transforming growth factor-β) superfamily and the IGF system modulate the response of these follicles to gonadotropins.

The TGF-β superfamily comprises over 30 structurally related but functionally diverse proteins that include two inhibins (A and B) and three activins (A, B and AB) (Knight and

Glister 2003). Inhibins consist of various dimeric glycoproteins (α and β subunits), which form molecular weight (MW) from 20->160kDa in bovine follicular fluid (Good, Weber et al. 1995) and negatively effect follicular FSH response (Silva, Groome et al. 1999). Inhibins also suppress estradiol production and neutralize activin actions that inhibit follicular growth and differentiation. Follicular fluid concentrations of inhibin A and inhibin B are increased and decreased with increasing follicle size, respectively (Knight and Glister 2003).

Activin is a dimer of the β inhibin subunits that opposes the actions of inhibins in both pituitary and ovary via its receptor. Activin activity is regulated by levels of follistatin, which neutralizes activin functions (Findlay 1993). Activin induces granulosa cell proliferation, and increases FSHr expression and steroidogenesis (Knight and Glister 2001). Activin-A concentrations increase during follicle growth, which is accompanied by a reciprocal decrease in follistatin (Austin, Mihm et al. 2001).

The ovarian IGF system comprises IGF-I and IGF-II, the IGF-type I receptor, and different insulin-like growth factor binding proteins (IGFBPs) and most recently protease(s) against the IGFBPs (Rivera and Fortune 2003). Free IGFs stimulate proliferation of follicular granulosa cells, and enhance estradiol, inhibin, and activin synthesis (Glister, Tannetta et al. 2001). Follicle oestrogenic activity is negatively correlated with intrafollicular amounts of lower MW IGFBPs (IGFBP-2,-4,and -5) (Echternkamp, Howard et al. 1994). IGF-II, type 1 IGF receptor and IGFBP-2,-3 and -4) have beean detected at the time of antrum formation in cattle (Armstrong, Baxter et al. 1998; Armstrong, Baxter et al. 2002). It is generally agreed that IGF-II is the major

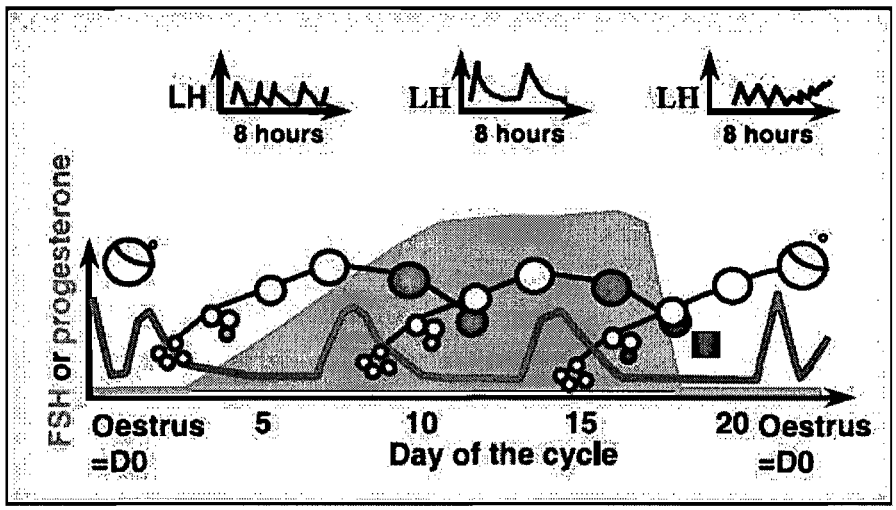

Figure 2: Gonadotropin regulation of dominant follicle turnover

Schematic diagram of the hormone environment and dominant follicle turnover in heifers with three dominant follicles per cycle; shaded follicles indicate onset of atresia.

(From Mihm, Crowe et al., 2002, Reprod Domest Anim. 37(4):191-200)

intra-follicular IGF ligand regulating the growth of bovine antral follicle (Webb, Campbell et al. 1999) via the type 1 IGF receptor (Lucy 2000). Further, in bovine dominant follicle fluid, decreased IGFBP-2 & -4 concentrations have been coupled to increases in estradiol levels (Mihm, Austin et al. 2000). The binding of IGFBPs to IGF is controlled by IGFBP proteases in follicular fluid, and IGFBP proteases levels are much higher in dominant compared to regressing subordinate follicles (Fortune, Rivera et al. 2001). The intrafollicular positive and negative feedback systems in the ruminant ovary are illustrated in Figure 3.

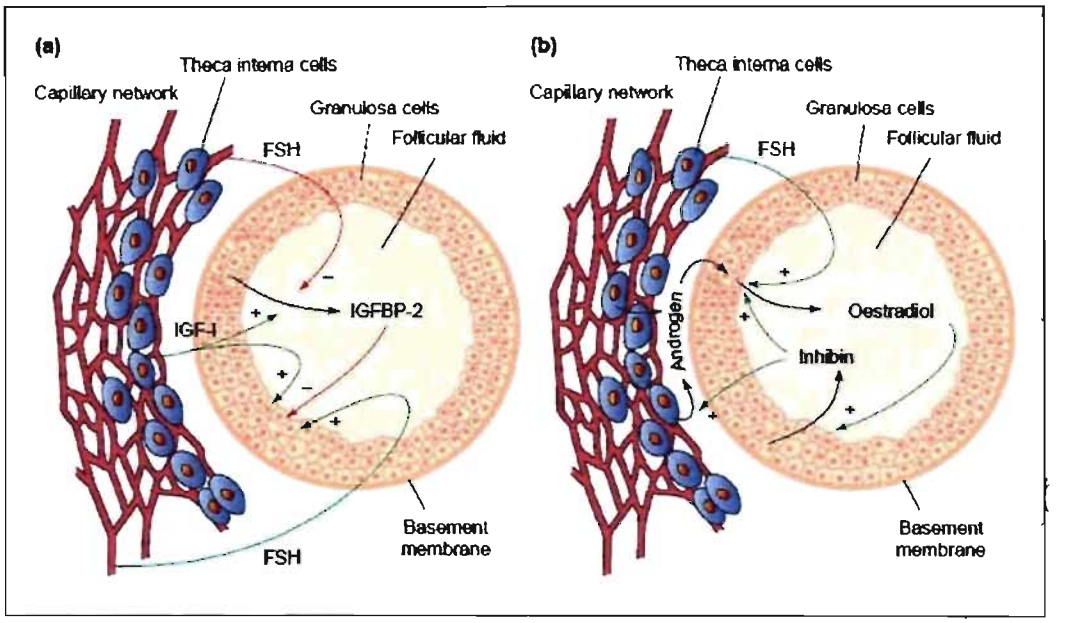

Figure 3: Intrafollicular positive and negative feedback systems

(a) The production of IGFBP-2 by GCs is regulated by IGF-I and FSH. The IGF-I enhanced FSH stimulation of GCs proliferation and differentiation is , in turn, inhibited by IGFBP-2. (b) Inhibin is stimulated by androgens and oestrogens. Insulin, in turn, enhances the FSH stimulation of estradiol by GCs.

(From Armstrong and Webb, 1997. Rev Reprod. 2(3):139-146)

FOLLICULAR STEROIDOGENESIS

One of basic functions of the ovarian follicle is the secretion of steroids. All steroids comprise a ring complex, made up of three cyclohexane rings (A, B, C) and a cyclopentane ring (D). The steroid hormones secreted by the ovarian follicle are be classified by the number of carbon atoms (C*n*) into progestins (C_{21}), androgens (C_{19}) and estrogens (C_{17}) (Gore-Langton and Armstrong 1994).

Steroidogenic pathways in the ovarian follicle

In follicles, all steroid sex hormones are derived from cholesterol (C_{27}), and then a series of steroid metabolites are produced by different steroidogenic enzymes; each enzyme converts one steroid to another. The major enzymes include three cytochrome P450 enzymes, P450 side-chain cleavage (P450scc), P450 17 α -hydroxylase ($P450_{17OH}$) and P450 aromatase (P450arom), and two hydroxysteroid dehydrogenases, 3 β-hydroxysteroid dehydrogenase (3 β-HSD) and 17 β-HSD (Sahmi, Nicola et al. 2006).

Intracellular cholesterol may be derived from de novo synthesis or cellular uptake of lipoprotein cholesterol. The majority of blood cholesterol is transported by either low density lipoproteins (LDL) or high density lipoproteins (HDL), depending on the animal species(Grummer and Carroll 1988). The predominant form used for steroidogenesis appears to be LDL which binds to the LDL receptor on follicle cells. While cholesterol is imported into the cell, it is maintained within lipid droplets as cholesterol esters, and is converted to free cholesterol by cholesterol ester hydrolase(Grummer and Carroll 1988). Free cholesterol is internalized into mitochondria by steroidogenic acute regulatory

protein (StAR), the rate-limiting step for steroidogenesis ((Stocco 2001).

The initial step of steroidogenesis is the conversion of cholesterol to pregnenolone. The conversion is catalysed by P450scc, which is present in the inner mitochondrial membrane in all steroidogenic cells (Farkash, Timberg et al. 1986). After pregnenolone is formed, it can be further metabolized by two different enzymes. The first pathway is conversion to progesterone by 3 β -HSD. The other pathway is metabolism to dehydroepiandrosterone by P450$_{17\text{-OH}}$. These two alternative pathways for the metabolism of pregnenolone are called the Δ-4 and Δ-5 pathways(Conley and Bird 1997), respectively. The pathway taken is species and tissue dependent. The enzyme P450$_{17\text{-OH}}$ is not expressed in ruminant luteal and GCs, so steroidogenesis goes through the Δ-4 pathway, resulting in progesterone biosynthesis, this progesterone is not metabolised further and is secreted. In theca cells, there is abundant activity, so pregnenolone goes through the Δ-5 pathway to dehydroepiandrosterone(Conley and Bird 1997). This dehydroepiandrosterone then undergoes conversion to androstenedione by 3β-HSD activity. Theca cells also convert androstenedione to testosterone with 17β-HSD, and these two androgens are secreted. In ruminant GCs, P450arom converts androstenedione to estrone, and then the estrone is converted to estradiol by 17β-HSD. Testosterone can be metabolised directly to estradiol by P450arom (Conley and Bird 1997). The biosynthetic pathway of steroid hormones is illustrated in Figure 4.

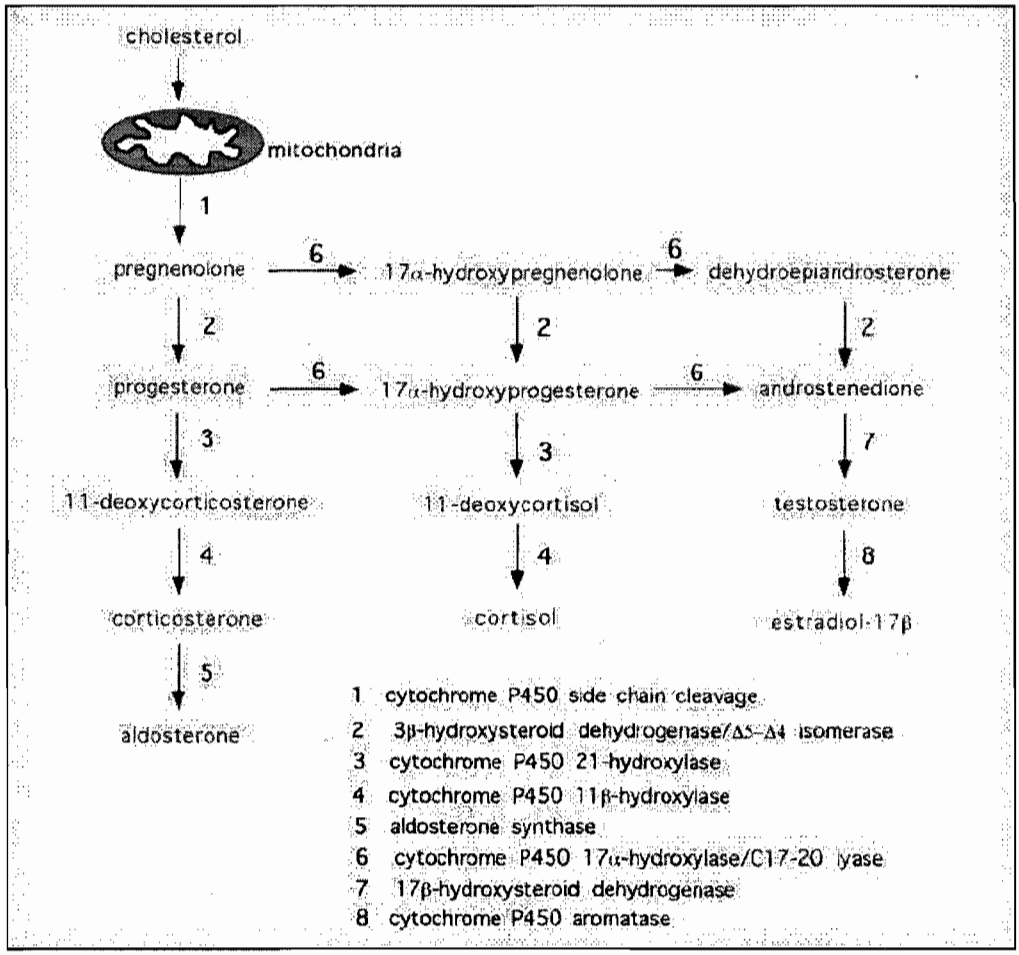

Figure.4. The biosynthetic pathway of steroid hormones.

Shown are the biosynthetic pathways leading from cholesterol to the major steroids produced in the adrenal, ovary, and testis. Conversion of cholesterol to pregnenolone and 11-deoxycortisol to cortisol occurs in the mitochondria; the remaining reactions occur in the microsomal compartment.

(From Stocco 2001, Annu Rev Physiol. 63:193-213)

Regulation of follicular steroidogenesis

Follicular steroidogenesis is dependent on the coordinated actions of FSH and LH with their receptors on ovarian follicles. The enzymes expressed in theca cells are regulated by LH. LHr are present on thecal cell at all stages of follicular development (Bao and Garverick 1998), and LHr activation acts via cyclic AMP to stimulate androgen production by increasing P450scc, P450c17 and 3 β -HSD expression/ activity. LH has also been shown to acutely upregulate StAR gene expression and LDL receptor mRNA levels. In granulosa cells, FSH stimulates P450scc, P450arom and 17b-HSD mRNA/activity (Garverick, Baxter et al. 2002) and LDL receptor levels, so FSH stimulates progesterone and estradiol secretion from granulosa cells. LHr is not expressed in granulosa cells until follicles reach 8 mm diameter (Bao and Garverick 1998), so LH stimulates progesterone secretion from dominant (and luteinized) granulosa cells.

Steroidogenesis is not only regulated by gonadotropins, as a number of growth factors also effect the expression / activity of steroidogenic enzymes. Components of the IGF system increase responsiveness of follicular cells to FSH and LH to increase secretion of follicular steroids (Hammond, Mondschein et al. 1991). In bovine granulosa cells, insulin/IGF-I play important roles in stimulating P450arom activity and mRNA levels and estradiol secretion from bovine granulosa cells in vitro (Silva and Price 2002). Insulin/ IGF-I and IGF-II stimulate progesterone and androstenedione secretion form theca cells, and the effects of IGF-II are mediated through IGF type I receptors (Spicer 2004). Another major group of growth factors is the transforming growth factor- β (TGF- β) family. TGF- β has been shown to enhance gonadotropin-stimulated steroidogenesis. Two other growth

factors have the opposite effect on steroidogenesis: the (EGF) epidermal growth factors (Hsueh, Welsh et al. 1981) and fibroblast growth factors (FGF) inhibit steroidogenesis (Spicer and Stewart 1996).

StAR protein is also important for the regulation of steroidogenesis. StAR mediates the transfer of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane (the rate-limiting step). In the ovary, StAR is expressed in theca (Pollack, Furth et al. 1997), granulosa (Kiriakidou, McAllister et al. 1996), and luteal cells (Pollack, Furth et al. 1997). StAR expression is up-regulated by LH (Juengel, Larrick et al. 1998; Wang, Walsh et al. 1999), insulin (Sekar, Garmey et al. 2000), FSH (LaVoie, Garmey et al. 1999), cAMP analogs (Balasubramanian, Lavoie et al. 1997; Mamluk, Greber et al. 1999), IGF-1 (Pescador, Stocco et al. 1999), insulin (Mamluk, Greber et al. 1999) and GDF-9 (Elvin, Clark et al. 1999). The exact mechanism of regulation of StAR expression and StAR mediated transfer of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane is not clear. A current model is as follows (Stocco 2001): trophic hormone (LH/ACTH) interaction with specific membrane receptors results in the activation of G proteins (G), which in turn, activate membrane-associated adenylyl cyclases (AC) that catalyzes cAMP formation from ATP. cAMP then activates PKA, which results in the rapid induction of StAR protein production and its plosphorylation. Once the protein of StAR is formed in cytosol, it will binds to the recognition site on the outer mitochrondial membrane, and cholesterol is transported from the cytosol to the inside of the mitochondria.

The Ca^{2+} messenger system and Cl^- ions are also involved in potentiating trophic

hormone-stimulated steroidogenesis and StAR expression. G protein or cAMP activate PLA2, and catalyzes AA release from phospholipids. cAMP also activates ARTISt (AA-related thioesterase involved in steroidogenesis); three enzymes: lipoxygenase (LPOX), epoxygenase (EPOX), or cyclooxygenase (COX). LPOX and EPOX metabolites play important roles in StAR expression and steroid synthesis, but COX2 plays an inhibitory role (Stocco, Wang et al. 2005). The PKC pathway also involved the regulation of StAR expression and steroidogenesis. Activation of the PKC pathway results in an increase in the transcription and translation of StAR, but not its phosphorylation. Thus, StAR induced through the PKC pathway is inactive in cholesterol transfer (Stocco 2001). Proposed model of multiple signaling pathways in the regulation of StAR expression and steroidogenesis is illustrated in Figure 5.

The role of steroids in follicular growth

The steroidogenic pathway within the ovary gives rise to progestins, androgens, and oestrogens, all of which act via specific nuclear receptors to regulate reproductive function and maintain fertility (Drummond 2006). Progestins are mainly secreted by the corpus luteum following ovulation, and are also secreted by large preovulatory follicles (Graham and Clarke 1997). Progestins secreted by the follicle are pregnenolone, which is precursor to all steroid hormones. Progestins play a limited role in follicular growth and development, its action confined largely to ovulation; the ovulatory process is progesterone dependent (Drummond 2006). Progesterone acts via its corresponding receptor, the progesterone receptor (PR). PR comprises two forms, PR-A and PR-B

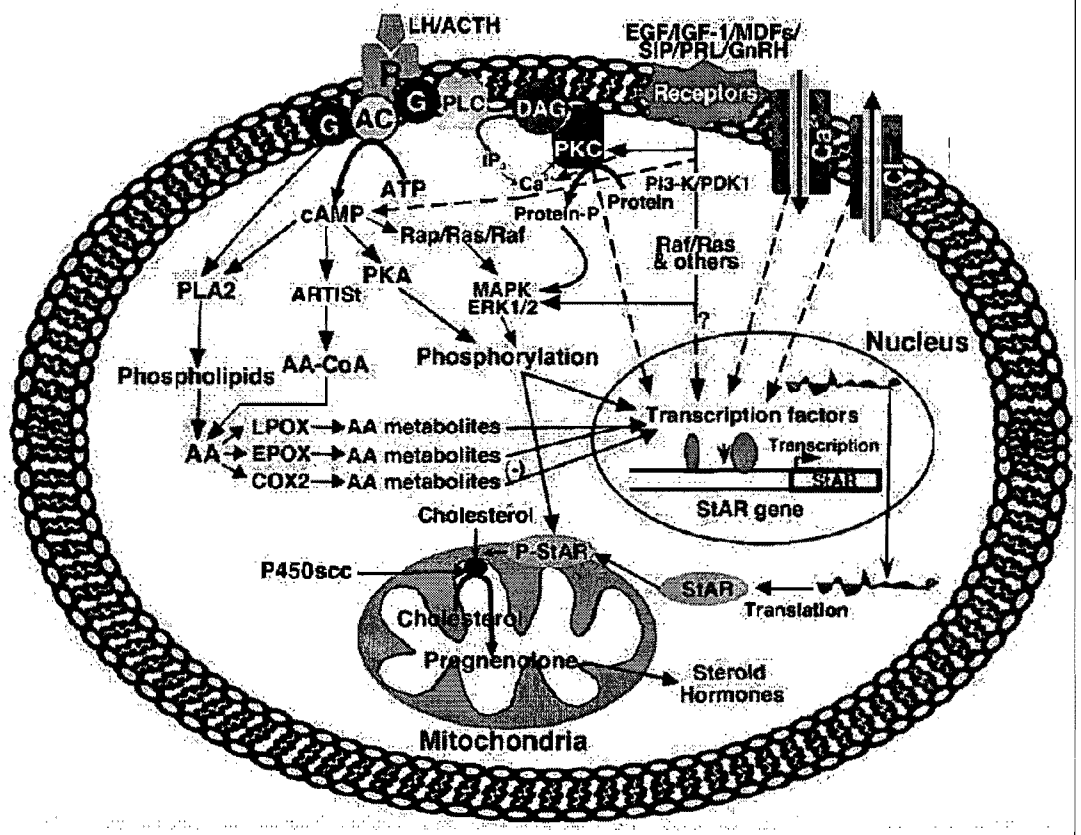

Figure.5. Proposed model illustrating of multiple Signaling Pathways in the regulation of StAR Expression and Steroidogenesis

(From Stocco, Wang et al., 2005, Mol Endocrinol. 19(11):2647-59)

(Kastner, Bocquel et al. 1990), but they arise form a single gene. The data from in vitro systems has demonstrated that PR-A and PR-B have different transcription activation properties when bound to progesterone (Conneely, Mulac-Jericevic et al. 2003) and PR-A is a dominant repressor of PR-B (Vegeto, Shahbaz et al. 1993). PR-A knock-out mice displays severe abnormalities in ovarian and uterine function, cause infertility. In contrast, in PR-B knock-out mice, the ovarian or uterine responses to progesterone are normal (Conneely, Mulac-Jericevic et al. 2003). Consequently, PR knock-out reveals that PR-A is necessary for ovulation whereas PR-B is not. PR mRNA was induced by LH in cultured porcine (Iwai, Yasuda et al. 1991) and rat (Natraj and Richards 1993) granulosa cells. These studies suggest that LH-induced PR is essential for ovulation and luteinisation.

Androgens, primarily androstenedione and testosterone, are produced by theca cells under LH control (Smyth, Miro et al. 1993). Androgen act via its receptor (AR), which is expressed in granulosa cells throughout follicle development in pigs, cattle and humans (Horie, Takakura et al. 1992; Smyth, Miro et al. 1995; Slomczynska, Duda et al. 2001; Hampton, Manikkam et al. 2004) but during late preovulatory development, transcription of the granulosa AR gene and AR protein levels decline (Hillier and Tetsuka 1997). Androgens have been shown to promote early follicular growth, but also impede follicular development by stimulating atresia and apoptosis (Drummond 2006). Many earlier studies have demonstrated that androgens enhance the differentiation of FSH-mediated granulosa cells by increasing progesterone and oestradiol secretion (Nimrod 1977; Hillier and De Zwart 1981; Hillier and Tetsuka 1997) and also regulating the action of IGF-I signaling system. IGF-I and IGF-I receptor mRNAs are enhanced by both testosterone and

dihydrotestosterone. Therefore, androgens play indispensable roles in follicle development, as female AR knockout mice have reduced fertility (Hu, Wang et al. 2004). One of the most important role for androgens in the ovary is the synthesis of oestrogen via P450arom.

Estrogens are secreted from granulosa cells in and are essential for folliculogenesis. Estrogen synthesis is first apparent in the late preantral stage when the follicle possesses all the components of the 'two cell, two gonadotrophns' model. Estrogen production is limited at the small antral stage because of a low ability to produce androgen substrate, although aromatase activity is present at this stage (Carson, Richards et al. 1981). Beyond the small antral stage, increased aromatase and androgen production increases follicular estrogen secretion (Hillier and De Zwart 1981). Increased FSH concentrations increase expression of P450arom (Hillier 1994). There are two forms of the estrogen receptor (ER) ERα and ERβ. ERα inhibits ovulation while ERβ stimulates follicular growth, decreases atresia, and enhances the number of oocytes released at ovulation (Drummond 2006). Female ERα knockout mice are infertile and ERβ knockout mice are fertile (Korach, Couse et al. 1996). Double ER knockout mice are distinct from the individual ER knockouts. Double ER knockout female adult mice exhibit follicle transdifferentiation to structures resembling seminiferous tubules of the testis, including Sertoli-like cells and expression of Müllerian inhibiting substance, sulfated glycoprotein-2, and Sox9. Therefore, both receptors are required for the maintenance of germ and somatic cells in the postnatal ovary (Couse and Korach 1999). Estrogen is a potent mitogen for rodent granulosa cells in vivo (Drummond 2006). The combined action of estrogen, FSH and LH increase cAMP levels, and also increase the number of cAMP binding sites in granulosa cell (Richards,

Jonassen et al. 1979).

In summary, steroid hormones play important roles in follicular development via specific receptors. In the absence of any single sex steroid, ovarian function and subsequently fertility are compromised.

Steroidogenesis in bovine follicular development

Ovarian steroidogenesis occurs according to the "two cell-two gonadotropin" theory. The "two cell-two gonadotropin" model suggests that both granulosa and theca cells are involved in production of estradiol (Bao and Garverick 1998). In this model, GCs contain FSHr and theca cells contain LHr (Havelock, Rainey et al. 2004; Drummond 2006). The biosynthetic pathway of steroid hormones in the ovary is illustrated in Figure 6a;b.

In cattle, preantral and early antral follicular development up to 2 to 4 mm in diameter is believed to be gonadotropin- independent. Some investigations indicate that the expression of FSHr mRNA can be localized within primary follicles with one to two layers of granulosa cells (Bao and Garverick 1998), but steroidogeneic enzymes do not appear until formation of the theca cell layer (Bao, Garverick et al. 1997); exogenous FSH increased the number of small preantral follicles in hyppophysectomized mice whereas follicles can growth to the antral stage in the absence of gonadotropic support (Camp, Rahal et al. 1991), these data suggest that FSH may stimulate granulosa cell proliferation and follicular development at early stages of growth. In secondary and early antral follicles, the theca cells start to express mRNA cording for LH receptors, P450scc, $P450_{17\text{-}OH}$ and 3β-HSD, and expression generally increases with growth of early antral

follicles (Bao, Garverick et al. 1997), thus follicles at early stages of development are able to make androgens. Nevertheless, the follicles during the early growing stage are not able to convert androgen to estradiol in granulosa cell because P450arom is not expressed in follicles <4 mm diameter (Bao and Garverick 1998). Moreover, follicles <4 mm in diameter do not express P450scc or 3β-HSD, so that granulosa cells are unable to convert cholesterol to pregnenolone and subsequently to progesterone. Therefore, thecal cells are the main source for follicular fluid steroid hormones at this stage of development (pregnenolone, progesterone, and androgen) (Bao and Garverick 1998).

Follicular development beyond 4 mm in diameter in cattle is considered to be gonadotropin-dependent, including recruitment, selection, dominance and ovulation. Previous studies have indicated that follicular recruitment is associated with increased expression of P450scc and P450arom in granulosa cells (Xu, Garverick et al. 1995). FSH is considered essential for follicular growth beyond 4mm and regulates the expression of P450scc and P450arom in granulosa cells during follicular recruitment (Adams, Matteri et al. 1992). During recruitment, follicles grow from 5mm to 8 mm and granulosa cells express P450arom and P450scc mRNA, but not 3 β -HSD mRNA (no 3 β -HSD mRNA was detected in GC of the recruited follicles <9mm) (Bao, Garverick et al. 1997), and theca cells express LHr, P450scc, $P450_{17\text{-}OH}$, and StAR mRNA. Thus granulosa cells at this stage of development are able to convert cholesterol to pregnenolone and androstenedione to estrone, but can't convert pregnenolone to progesterone (Bao and Garverick 1998).

In cattle, a single follicle is selected from a cohort of medium-sized growing follicles

and becomes the dominant follicle. The dominant follicles are characterised by induction of expression of LHr and 3 β -HSD in granulosa cells, thus granulosa cells are able to convert not only thecal androgen to more estradiol, but also convert cholesterol to pregnenolone and progesterone.

During the growth phase of the dominant follicle, expression of mRNA for gonadotropin receptors, steroidogenic enzymes and StAR increases in the thecal and/or granulosa cells (Bao and Garverick 1998), so the dominant follicle is able to produce higher amounts of estradiol. It is known that small follicles contain relatively little estradiol; follicular fluid estradiol concentrations increase with follicle size in healthy growing follicles, and decrease in subordinate follicles and in regressing dominant follicles (Price, Carriere et al. 1995). Thus estradiol concentration is a marker for the degree of health/atresia of follicles. The regresses of subordinate follicles are associated with decrease in all steroidogenic enzymes in granulosa cells.

Dominant follicle atresia is also associated with changes in steroidogenic enzymes; the first changes occurs as the follicle reaches the 'static' phase (day 4 and 6 of the follicle wave) of its growth phase when there is a reduction in mRNA for P450scc, P450$_{17\text{-OH}}$, and LHr in theca cells, and P450scc in granulosa cells (Bao and Garverick 1998), at this stage, dominant follicles secrete less estradiol. When the dominant follicle starts to regress, no more decline of steroidogenic enzyme mRNA occurs in the theca cells, but a loss of P450scc, P450arom, LHr and 3 β -HSD occurs in granulosa cells (Bao and Garverick 1998).

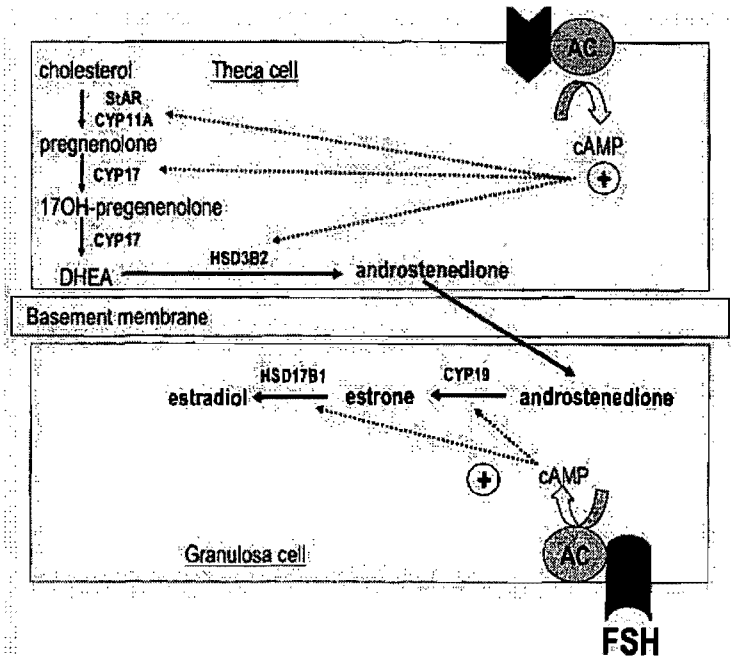

Figure.6a. Follicular phase steroid biosynthesis in the ovary with the illustration of the two-cell/two-gonadotropin theory

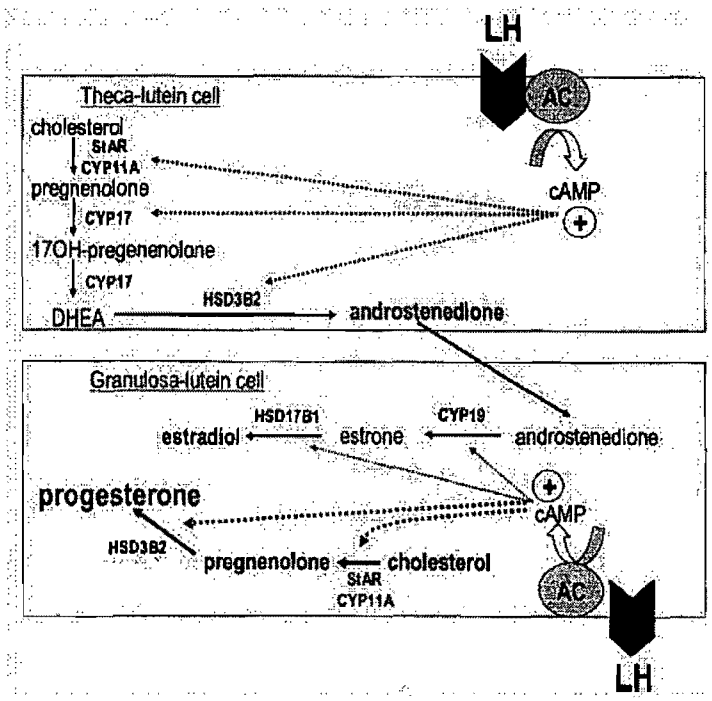

Figure.6b. Steroidogenic pathways for luteinized theca and granulosa cells.

(From Havelock, Rainey et al., 2004, Mol and Cell Endocrinol 228(1-2):67–78)

AROMATASE

The structure of aromatase and expression

Aromatase (P450arom) is the product of the Cyp19 gene. The aromatase enzyme complex consists of two components, P450arom and the redox partner protein NADPH-cytochrome P450 oxidoreductase (reductase). Both components are highly conserved between vertebrates (Conley and Hinshelwood 2001). The amino acid sequences from bovine, rat, mouse, chicken and trout P450arom are 86%, 77%, 81%, 73%, and 52% homologous to the human, respectively (Simpson, Mahendroo et al. 1994).

The catalytic process leading to the aromatization of androgens requires the sequential transfer of three pairs of electrons and consumes three moles of oxygen and three moles of reduced NADPH in the synthesis of one mole of estrogen. The reaction involves two consecutive hydroxylations at the C19 methyl group (19-hydroxylase activity) of the steroid substrate, forming 19-hydroxy and eventually 19-oxo-intermediates. The third oxidative event, which is still debated, culminates in the cleavage of the angular C19 methyl group (19-demethylase or desmolase activity, aromatization of the steroid A ring and estrogen formation (Conley and Hinshelwood 2001). The catalytic process leading to the aromatization of androgens is illustrated in Figure 7.

Although P450arom expression occurs in the gonads and the brain of most vertebrate species, it is also expressed in the placenta, adipose tissue, liver, skin and bone (Boerboom, Kerban et al. 1999; Conley and Hinshelwood 2001). In the ovary, P450arom is expressed in granulosa cells, which is much higher in preovulatory follicles than in small follicles (Hickey, Chen et al. 1988). In some species, such as human and rodents, P450arom is also

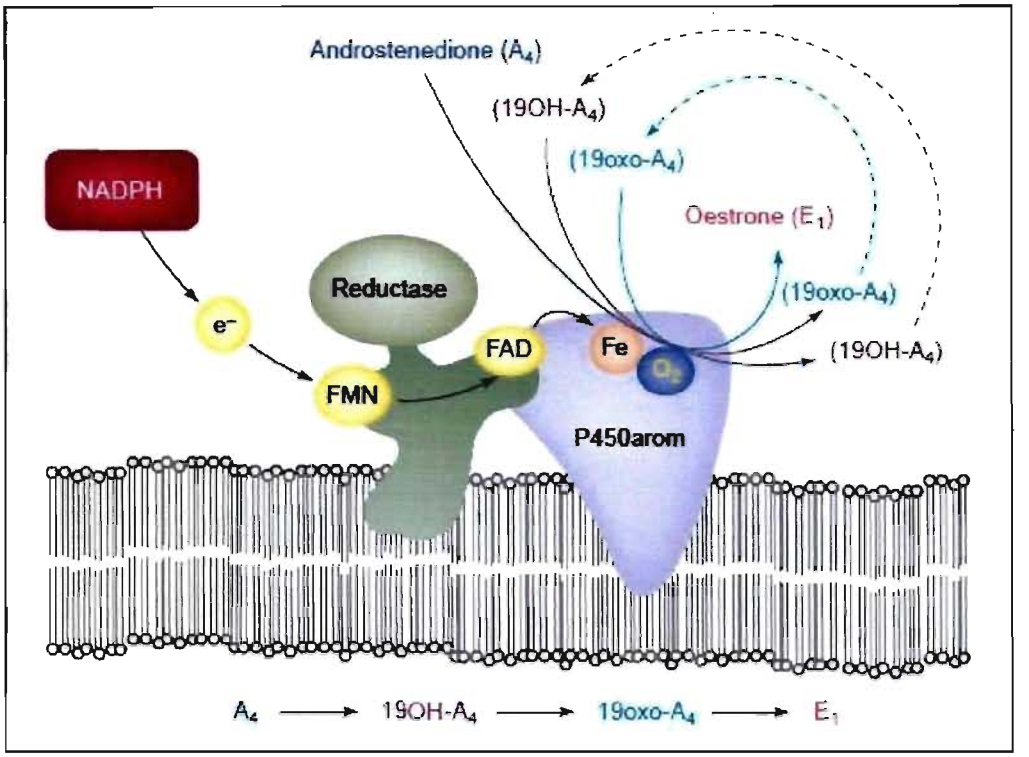

Figure.7. The catalytic process leading to the aromatization of androgens

The microsomal membrane anchoring the aromatase enzyme complex which consists of aromatase cytochrome P450 (P450arom) and the redox partner protein NADPH-cytochro--me P450 oxidoreduxtase (reductase). The flavin prosthetic groups of the reductase, flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), are indicated.

(Modified from Conley and Hinshelwood 2001. Reproduction. 121(5):685-95)

localized to the corpus luteum (Doody, Lorence et al. 1990), and in others such as the pig in theca interna (Conley and Bird 1997). In the testes, P450arom activity is found in sertoli cells, leydig cells (Abney 1999), and it also expressed in testicular germ cells (Janulis, Hess et al. 1996). In both the ovary and testes, gonadotropins act through increasing concentrations of intra-cellular cAMP to induce expression of P450arom. In the brain, high P450arom expression was found in the medial basal hypothalamic area and amygdaloid region of male rat (Lephart 1996).

The regulation of Cyp19 expression occurs, in part, through the use of tissue-specific promoters and by alternative splicing mechanisms (Conley and Hinshelwood 2001). In human placenta, the expression is controlled by promoter I.1, which lies at least 40kb upstream from the start site of transcription (Kamat, Graves et al. 1999). In liver, the promoter lies about 15kb upstream of the start site of translation (promoter 1.4). In contract, promoter II driving transcription in the ovary lies just upstream of the start site of the transcription (Means, Kilgore et al. 1991). Although, expression of P450arom is driven by different promoters, these first exons are spliced onto a common splice site of the codon in exon II that initiates translation, so the protein expressed in the different tissues is identical (Simpson, Mahendroo et al. 1994).

The structure of Cyp19 gene

Many investigations of the gene encoding P450arom in human and other mammals (with the notable execption of the pig) have demonstrated that P450arom is encoded by the single gene Cyp19. Therefore, P450arom expressed only one protein with one amino

acid sequence in different tissues. There is high degree of sequence conservation among the tissue-specific expression, particularly evident in the gonad- and brain- specific first exons and promoters (Conley and Hinshelwood 2001) In most species, Cyp19 is composed of nine coding exons (exons 2-10) (Hanoux, Bouraima et al. 2003). Alternative promoter use results in transcript variants with different 5'untranslated regions (5'UTR) (Simpson, Mahendroo et al. 1994).

The bovine aromatase gene (Cyp 19) has been mapped to band q2.6 of chromosome 10 in cattle (Goldammer, Guerin et al. 1994), and comprises at least 14 exons (1.1, 1.2a, 1.2b, 1.3, 1.4, and 2-10 coding exons) spanning more than 56 kb of genomic DNA. The open reading frame (ORF) comprises 1509 bp and shows 87%, 78% and 78% sequence homology to the coding areas of the human, rat and mouse genes, respectively. The 3'-untranslated region (3'-UTR) of bovine Cyp19 transcript extends over 3486 bp, about 2 kb longer than human Cyp19 gene. There are six promoters in the bovine Cyp19 gene (Vanselow and Furbass 1995).

In bovine ovarian granulosa cells, all transcript variants have been detected with RT-PCR (Furbass, Kalbe et al. 1997), the most important promoter being promoter 2 (P2), which is the most proximal promoter of the Cyp19 gene. The steroidogenic factor-1 (SF1) binding motif occurs in P2, therefore this might confer cAMP responsiveness on the bovine P2 (Lala, Rice et al. 1992; Furbass, Kalbe et al. 1997). Another promoter adjacent to exon 1.1 (P1.1) contributes considerably to Cyp19 expression in granulosa cells. P1.1 and P2 derived transcripts were found highly correlated with total expression of Cyp19 (Lenz, Pohland et al. 2004). Both promoters are co-regulated although at different levels

of activity and that not only P2 but also P1.1 is involved in the regulation of Cyp19 expression in bovine granulosa cells (Lenz, Pohland et al. 2004). Other transcripts derived from P1.2, P1.3, P1.4 and P1.5 have low expression patterns in granulosa cells, suggestion that these promoters are not involved in the up-regulation of Cyp19 expression during recruitment and selection. In the developing follicles, P2 has been considered the pivotal promoter, but after luteinisation, P2 is switched off and the transcripts are almost entirely derived from promoter P1.1. Therefore, promoter P1.1 drives Cyp19 expression in corpus luteum (Lenz, Pohland et al. 2004).

In placenta, unlike in the ovary and the brain, 5'UTRs of Cyp19 transcripts do not show any sequence similarities among species (<40%) (Simpson, Mahendroo et al. 1994; Hinshelwood, Liu et al. 1995). Even species as closely related as cows and sheep express different transcripts in the placenta (Vanselow, Zsolnai et al. 1999). In cattle, 6 transcript variants were found in placenta, including those comprising exons 1.1, 1.2a, 1.2b or 1.3 although the most abundant was the variant comprising exon 1.1 (Furbass, Kalbe et al. 1997).

In bovine brain, exon 1.4 is the prominent first exon variant, and the nucleotide sequence of it is homologous to brain-specific transcripts in primates and rodents (Honda, Harada et al. 1994). Therefore, brain-specific Cyp19 expression driven by the promoter upstream from exon 1.4 is of functional importance.

Regulation of Cyp19 mRNA stability

Control of gene expression occurs at multiple levels, in cluding transcription, RNA

processing, mRNA nuclear export and localization, mRNA decay, translation and posttranslational events. The regulation of protein production is principally achieved by controlling mRNA levels. The stability of a particular mRNA is controlled by specific interactions between is structural elements and RNA-binding proteins that can be general or mRNA-specific. Regulated mRNA stability is achieved through fluctuations in half-lives to developmental or environment stimuli like nutrient levels, cytokines, hormones (Guhaniyogi and Brewer 2001). Levels of mRNA encoding P450arom are upregulated in rodent, human, and bovine granulosa cells by FSH (Steinkampf, Mendelson et al. 1987; Fitzpatrick and Richards 1991; Manuel Silva and Price 2000; Garverick, Baxter et al. 2002) and insulin/insulin-like growth factor-1 (Steinkampf, Mendelson et al. 1988; Silva and Price 2002). In contrast, LH has been shown to downregulate P450arom mRNA levels in rats and cattle (Voss and Fortune 1993; Fitzpatrick, Carlone et al. 1997; Komar, Berndtson et al. 2001).

How much control of P450arom mRNA abundance is exerted at the transcriptional compared with the translational levels is not clear. But several studies have indicated that the half-life of Cyp19 mRNA is 3-7 hours, depending on species (Sahmi, Nicola et al. 2006), although it is longer in humans (12h). The translation inhibitor cycloheximide stabilized P450arom mRNA in rat and bovine granulose cells (Fitzpatrick, Carlone et al. 1997; Sahmi, Nicola et al. 2006), suggesting that Cyp19 mRNA abundance might also be regulated at a post-translational step.

The regulation of mRNA stability is complex, the 3'-untranslated region (3'UTR) of many mRNA plays important role in mRNA degradation (Staton, Thomson et al. 2000).

3'UTR of bovine Cyp19 mRNA is approximately 3 kb lager than that in human (Means, Mahendroo et al. 1989; Doody, Lephart et al. 1991; Hinshelwood, Corbin et al. 1993), and it contains 9 AUUUA and AUUUUA motifs (Sahmi, pervious studies). AU-rich elements (ARE) within 3'UTR are main determinants of mRNA stability, which interact with specific RNA binding proteins in *trans* to stabilizer or destabilize mRNA.

Although post-transcriptional regulation of Cyp19 mRNA has yet to be demonstrated, there is evidence that a protein or protein complex specifically binds to the bovine Cyp19 3'UTR (Sahmi, pervious studies).

HYPOTHESIS AND OBJECTIVE

The mRNA encoding the bovine aromatase gene (Cyp19) has a long (3.5kb) 3'-UTR and a short half life (3h). Previous studies demonstrated that the full length P450arom 3'-UTR binds to a protein (or protein complex) found in granulosa cells, but not in corpus luteum, lung, liver, kidney, pancreas or bladder extracts. We hypothesize that posttranscriptional regulation may play an important role for control of P450arom expression and therefore enzyme activity.

The objective of this study was to purify the putative Cyp19 3'-UTR binding protein.

MATERIALS AND METHODS

(Unless otherwise stated, all reagents were purchased from Sigma-Aldrich)

Protein preparation

Total cell protein was extracted from bovine granulosa cells from follicles 2-6mm diameter with lysis buffer (10mM Hepes (Invitrogen) PH 7.6, containing 40mM KCL, 3mM $MgCl_2$, 2mM DTT (GE Healthcare), 5% glycerol, 0.5% Nonidet P40 (ICN Biomedicals), 0.5 mM phenyl-methylsulfonylfluorde (PMSF) and 8 μg/ml leupeptin). The sample was placed on ice for 15min. Protein extracts were clarified by centrifugation at 3000 g at 4 ℃ for 10 min.

Total bovine granulosa cells proteins were pre-purified by heparin-agarose beads (heparin agarose type I), which are used for isolation of DNA/RNA-binding proteins. Heparin agarose beads (500μl) were washed with buffer A (20mM Hepes pH 7.8, containing 12.5mM MgCl2, 0.5mM EDTA, 20% Glycerol, 40mM KCl) 5 times. Supernatants were discarded after centrifugation of beads at 3000 rpm for 5 seconds. Total cell proteins (500 μl) were added to the heparin-agarose beads, and incubated in a rotating incubator for 30 min at 4 ℃. Un-bound proteins were eluted by centrifugation for 5 sec at 3000 rpm. The beads were washed 5 times with wash buffer at 3000 rpm for 5 sec, and binding proteins were eluted with buffer B (20mM Hepes pH 7.8, containing 12.5mM MgCl2, 0.5mM EDTA, 20% Glycerol, 0.5M KCl). The supernatants were stored at -84℃.

Enriched RNA binding proteins were desalted using a Millipore centrifugal filter device with a molecular mass cutoff of 10 KDa (Millipore). Protein samples were brought

up to a volume of 2ml in buffer A, applied to the centricon membrane, and centrifuged at 4000X g for 30min. The concentrated sample was again diluted to 2ml in buffer A, applied to the membrane, and centrifuged at 4000X g for 40 min to a final volume of 30~50µl. All protein concentrations were measured with the Bradford protein assay (BioRad).

In Vitro Transcription

Radiolabeled RNA probes were generated by in vitro transcription using T7 RNA polymerase (Riboprobe In Vitro Transcription System; Promega) and the pGEM-T Easy plasmid containing a 1kb fragment encompassing +592 to +1714 of the noncoding region of bovine Cyp19 exon 10 previously demonstrated to bind to cell protein (Sahmi, previous studies) as template (3-UTR). A final reaction volume of 20µl contained 100 units of T7 polymerase, 1.1µl each of 10mM ATP, GTP, CTP and UTP (Invitrogen), 5µl αP^{32}-UTP (800ci/mmol; Perkin-Elmer), 4.4µl 5x transcription buffer (Invitrogen), 2.2µl 10mM DTT, 28 units RNAse inhibitor (Invitrogen) and 1µg cDNA template. Transcription reaction were incubated for 2h at 37 ℃, digested with 20units DNase (RQ1 DNase; Promega) for 20min at 37 ℃, then purified with RNAeasy mini kit (Qiagen). Specific activities of radiolabeled RNA probes were measured by liquid scintillation analyzer (TR1-CARB 2100TR, Packard; Bioscience).

Biotinylated RNA probes were generated as for radiolabeled probes. The final reaction was as above except 10mM Biotin-14-CTP (Invitrogen) replaced the αP^{32}-UTP. The concentrations of transcripts were determined by NanoDrop 1000 (Thermo Scientific).

To optimize capture of the biotinylated probe, RNA transcripts were generated in the

presence of 0%, 12%, 25%, 50% biotin-14-CTP and a fixed amount of P^{32}. Dual-labeled probes were incubated with 50µl streptavidin beads, and un-bound probes were by washing for 3 times. Radioactivities of dual-labeled probes, un-bound probes, washings and the bead-probe complex were measured by liquid scintillation counting.

Electrophoretic mobility shift assay (EMSA)/ UV cross-linking

Typically, 20µg proteins were incubated with 14 units of RNAse inhibitor, 2µg tRNA, 10µg heparin sulfate and 4-6 x 10^6 cpm of P^{32}-labeled riboprobe (denatured first for 5min at 95 ℃) in final volume of 20µl at room temperature for 40 min. The RNA-protein complexes were cross-linked on ice by exposure to UV light at 999mJ for 15min in a GSGenelinker (BioRad), followed by incubation with 10 units RNAse One Ribonuclease for 20min (Promega). The UV cross-linked products were separated on a 10% SDS-polyacrylamide gel and detected by autoradiography with a phosphorimager (Molecular Dynamics Storm 840; GE Healthcare) and quantified using NIH Image software.

Isolation of RNA-Binding protein

Several approaches were taken to isolate the RNA-binding protein; capturing the protein-probe complex to solid support via a biotinylated oligonucleotide linker, illustrated in Fig 8a; direct or indirect capture of biotinylated RNA probes onto solid support, as illustrated in Figure 8b; and capture of cross-linked complexes.

Fig. 8a: Biotinylated Oligonucleotide Capture

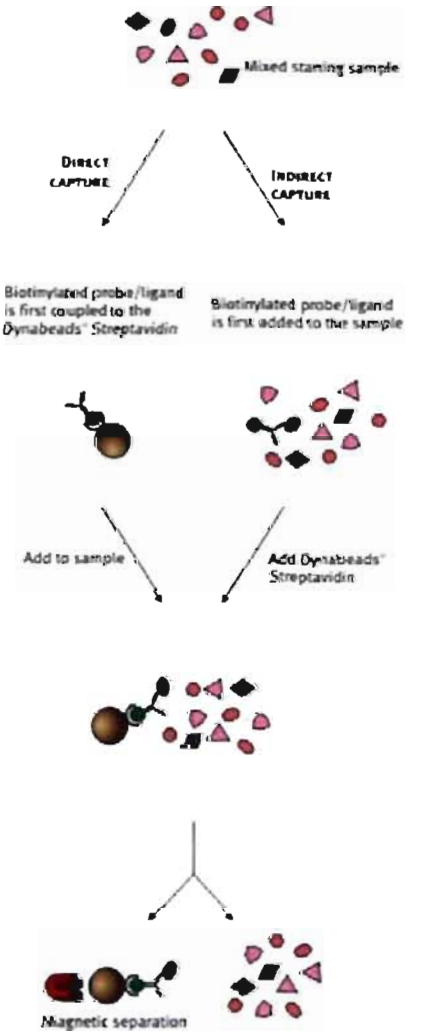

Fig. 8b: Biotinylated RNA probes direct and indirect capture

(From Dynabeads streptavidin products and applications; Invitrogen)

Biotinylated Oligonucleotide Capture

Biotinylated oligonucleotide (antisense: ACT AGT ATT CCT GCT TCT CCT TTA C-Biotin, 50nmol; Invitrogen) was hybridized to 50nmol non-labeled mRNA transcripts (denatured 5 min at 95 ℃ first) in a total volume of 100µl reaction buffer (10mM HEPES, 50mM KCl, 10units RNase inhibitor) for 1h at 37 ℃ with constant rotation. A volume of 100µl streptavidin beads (MagnaBind; PIERCE) were washed five times with 1ml of binding buffer (10mM HEPES pH 7.6; 5mM $MgCl_2$, 40mM KCl, 1mM DTT, 5% glycerol, 5mg/ml heparin), and the beads were added to the oligonucleotide - mRNA complex, and incubated for an additional 1h at room temperature with constant rotation. The beads were then washed for 5 times with 1ml binding buffer, and magnetically isolated (DynaMag™; Invitrogen). Total cell proteins or pre-purified RNA binding proteins (20µg) were mixed with the Magnabead-labeled mRNA probe, and incubated for 1h at room temperature with constant rotation. Specific RNA-binding protein was eluted from the beads with a high ionic strength salt and low pH elution buffer (1M NaCl in binding buffer, pH3.5). The eluate was boiled with SDS-PAGE loading buffer for 4min and separated on a 10% SDS-polyacrylamide gel, and silver-stained.

Direct capture of biotinylated RNA probes linked to solid support

Approximately 20units of RNase inhibitor was added to 1500µg biotinylated RNA probe and incubated with 500µl streptavidin beads at room temperature for 2h with constant rotation. Streptavidin beads were washed with binding buffer for 5 times before use. 100µg protein extracts were incubated with biotinylated RNA probe- streptavidin

bead mixture, and incubated for an additional 2h at room temperature with constant rotation. The beads were then washed ten times with 1ml of binding buffer at room temperature, and bound proteins were eluted from the beads with a high ionic strength salt and low pH elution buffer (pH 3.5), or boiled with 2x loading buffer for 4min.

Indirect capture of probe-protein complex to solid support

Approximately 20units of RNase inhibitor was added to 100μg proteins and incubated with 1500μg biotinylated RNA probe (denatured first for 5min at 95℃) for 2h with constant rotation. 500μl streptavidin beads were washed with binding buffer for 5 times, added to protein-biotinylated RNA probe mixture for an additional 2h at room temperature with constant rotation. The beads were then washed 10 times with binding buffer, and specific bound proteins were eluted by magnet with high ionic strength salt and low pH elution buffer (pH 3.5), or boiled with 2x loading buffer for 4min.

Capture of crosslinked complexes

50μg proteins were incubated with 14 units of RNAse inhibitor, 2μg tRNA, 10μg heparin sulfate and 1000μg of biotinylated RNA probe (denatured first for 5min at 95 ℃) in final volume of 20μl at room temperature for 40min. The mixture was cross-linked on ice by exposure to UV light at 999mJ for 15min in a GSGenelinker (BioRad), followed by incubation with 10units RNAse One Ribonuclease for 20min (Promega). Washed streptavidin beads were added to UV cross-linked products for 2h at room temperature with constant rotation. The beads were then washed 10 times with binding buffer, and

specific bound proteins were eluted by magnet with high ionic strength salt and low pH elution buffer (PH3.5), or boiled with 2x loading buffer for 4min.

Specific RNA binding proteins were separated on a 10% SDS-polyacrylamide gel, and first detected by Coomassie blue. If no protein was evident, silver staining was used for protein detection.

2-D Electrophoresis

1. Sample preparation: for protein identification experiment, samples were desalted first, by using a Millpore Centricon centrifugal filter device with a molecular mass cutoff of 10 kDa. Specific RNA binding proteins were brought up to a volume of 2ml in binding buffer, and centrifuged at 4500rpm for 30min, applied to the Centricon membrane, and centrifuged at 4500rpm for 40min to a final volume of 30~50μl. Protein concentrations were measured with the Bradford protein assay (BioRad).

2. Isoelectric Focusing: 100ng protein samples were add to the rehydration solution (8M urea (GE Healthcare); 2% CHAPS (GE Healthcare); 0.5% IPG buffer [pH 3-10] (GE Healthcare); trace of bromophenol blue; 7mg/2.5ml DTT before use). Mixtures were applied to 11-cm Immobiline DryStrip PH 3-10 (GE Healthcare) by using Immobilin Drystrip reswelling tray (GE Healthcare) at room temperature overnight (16h), covered in mineral oil. After rehydration, the samples were then separated on a Multiphore II flatbed system (GE Healthcare) for 16h at 15 ℃. The voltage was 300v for the first 3h, from 300

to 2000 V during the following 5h, and finally 8h at 2000 V.

3. Second Dimension: after focusing the dry strip was equilibrated 10min in equilibration buffer I (0.5M Tris/HCL pH6.5; 3.6g urea; 3ml glycerol; 0.1g SDS; 25mg DDT; and distilled water up to 10ml) and another 10min in equilibration buffer II (0.05M Tris/HCL pH6.5; 3.6g urea; 3ml glycerol; 0.1g SDS; 0.45g iodoacetamide, and distilled water up to 10ml). The second dimension was performed after placing the strip on ExcelGel SDS, gradient 8-18 (GE Healthcare) using the MultiPhor II flatbed system at 15 ℃ with 600 V (until bromophenol blue front reaches the anodic buffer strip).

4. Protein spot detection: after the gel was run, it was immediately immersed in fixing solution (30% ethanol and 10% glacial acetic acid in deionized water) and stained with silver nitrate (Silver Staining Kit, GE Healthcare).

Protein identification

Protein bands were cut from the gel and transferred to Institute for Research in Immunology and Cancer (IRIC) at University of Montreal. Samples were analyzed by Liquid chromatography-mass spectrometry (LC-MS) analysis.

RESULTS AND DISCUSSION

UV-crosslinking experiments were performed to determine whether the P450arom 3'UTR is associated with a *trans*-acting protein. The P450arom 3'UTR riboprobe formed a RNA-protein complex with cell extracts from bovine granulosa cells that migrated on SDS-PAGE gels at approximately 70 and 97kDa (Figure 9).

To identify which protein(s) bind to the P450arom 3'UTR, multiple techniques were used to isolate the binding complex. First, a biotinylated oligonucleotide linker was hybridized to the 3'UTR RNA, and this was incubated with GC protein extract. The complex formed was magnetically isolated on streptavidin beads and eluted with a high ionic strength salt buffer. After protein migrated on SDS-PAGE gels, no band was detected with Coomassie blue or silver stain. In conclusion, no useful proteins were isolated with this approach.

One problem with the approach taken above is that the amount of binding protein in total cell extracts might be to low to detect on gels. Accordingly, we attempted to concentrate the binding protein. DNA binding proteins are capable of binding to heparin, therefore we used heparin-agarose beads to enrich RNA binding proteins. Total protein extracts were incubated with heparin-agarose beads, and RNA binding proteins were eluted with high salt elution buffer. The data showed that 0.5M KCL elutes most binding proteins from heparin-agarose beads (Figure 10) and the protein profile differed between whole cells lysate and enriched RNA binding proteins (Figure 11). Subsequently, UV crosslinking experiments were performed to determine whether the RNA binding protein which eluted from the heparin-agarose beads were capable of binding to P450arom 3'UTR.

P450arom 3'UTR riboprobe formed a RNA-protein complex with enriched RNA binding protein that migrated on SDS-PAGE gels at approximately 70kDa and 97kDa (Fig 12).

For protein complex capture onto biotinylated probes, we first determined the optimum amount of biotin-CTP to use in probe generation. As shown in Table 1, 25% biotin-CTP provided maximum binding of dual labeled probe to the solid support, therefore this level of biotinylation was used in the remaining experiments. In direct capture experiments, biotinylated 3'UTR RNA probes were linked first to streptavidin beads, the enriched RNA binding protein extract was added, and after washing, binding proteins were eluted with high ionic strength salt and low pH elution buffer and separated on 10% SDS-PAGE gel. Multiple proteins spanning wide range molecular weights were detected by silver stain (Fig 13). In the indirect capture approach, biotinylated P450arom 3'UTR RNA probes were first incubated with enriched RNA binding protein and the complexes were isolated on streptavidin beads. Fewer proteins were isolated with this approach, in particular the larger molecular weight proteins were not detected (Fig 13). Thus, biotinylated RNA probes capture is generally useful for isolation of P450arom binding proteins, but as the number of proteins eluted was higher than those identified after UV cross-linking (EMSA), we believe that some proteins are non-specific (possibly RNases). Alternatively, the RNA-protein complex contains multiple proteins, and these resolve as individual proteins after elution from solid support under high salt concentrations.

As UV crosslinking forms irreversible RNA - protein complexes, we hypothesized that crosslinking may permit elution of intact complexes after capture on solid support. To

capture crosslinked complexes, biotinylated RNA probes were incubated with enriched RNA binding proteins, UV crosslinked, and RNase was used to digest unprotected RNA. Streptavidin beads were added to the reaction, and RNA-protein complexes were eluted from the beads. P450arom 3'UTR RNA binding proteins were migrated on 10% SDS-PAGE gel and clear bands were detected by silver staining. This resulted in much clearer bands (Figure 14), but still more bands were detected than in regular UV cross-linking (EMSA) studies. This may be due to non-specific RNA binding proteins (such as RNases) or enrichment of low-abundance proteins not previously detected. The bands numbered 1 - 4 in Fig 14 were excised from the gel and sent for identification by LC/LC-MS, however no clear identification was made. This is likely owing to the presence of variously sized strands of RNA irreversibly linked to peptide fragments, which would have altered the LC-MS profile of these fragments and render identification impossible.

The identification of RNA-binding proteins is a challenging task, confounded by the presence of ubiquitous and abundant non-specific binding proteins including RNases and co-factors, in the face of low-abundance and possibly low-affinity specific binding proteins. In addition, the RNA-protein complex that binds to the Cyp19 3'UTR may comprise multiple binding proteins, based on the number of bands observed. It is likely that protein complexes need to form to permit binding to the RNA, or the direct binding of one protein is required to recruit the binding with others. These factors complicate the strategy required for isolation of the protein(s).

Based on the above work, several strategies can be proposed for identification of the

protein. Owing to the apparent low-abundance of the binding proteins(s), it is important to start with an enriched extract of high protein concentration. Second, reversible cross-linking with formamide may permit capture of the complex, followed by removal of the probe and analysis of proteins. Third, the proteins released from the complex should be resolved on 2D-gels for higher purity and clearer identification. We attempted to run 2D-gels on eluted proteins, but the apparatus was malfunctioning (data not shown).

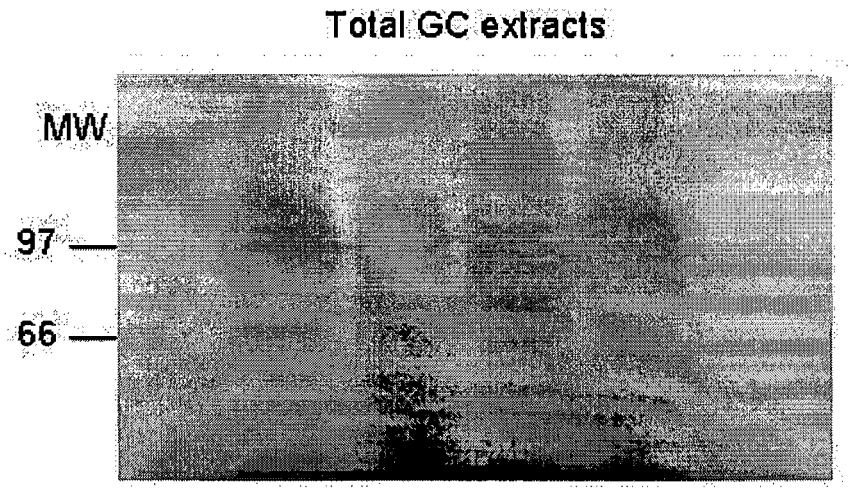

Fig 9. Radiolabeled P450arom 3'UTR RNA binding to protein extracts (20μg) from bovine granulosa cells (2-6mm). The riboprobe bound to the proteins of approximately 66kDa and 97kDa.

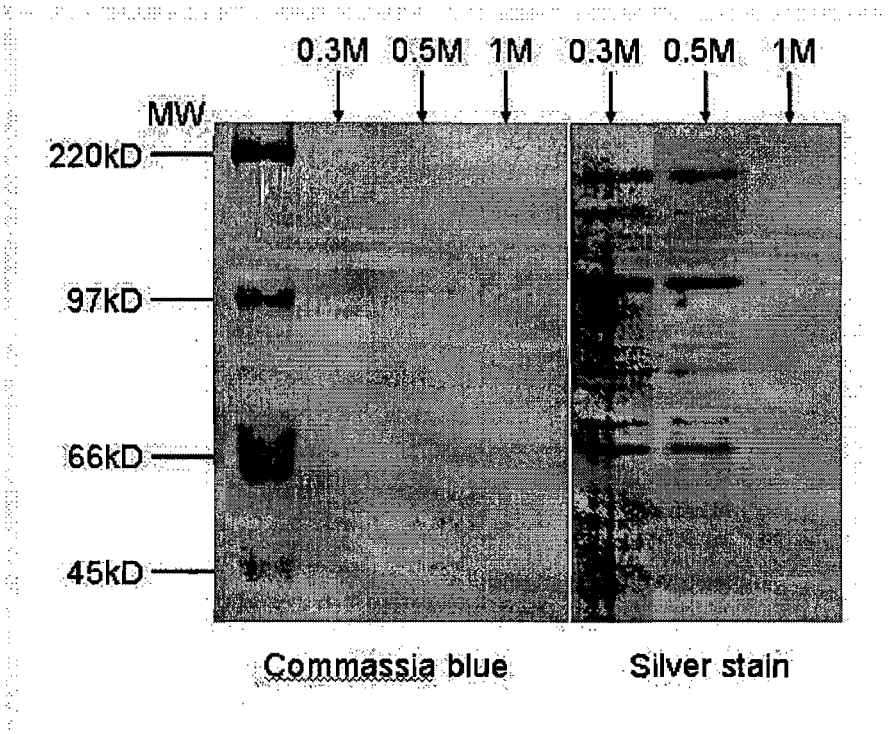

Fig 10. Heparin-agarose beads were used to enrich RNA binding proteins. Total GCs proteins were incubated with heparin-agarose beads, and RNA binding proteins were eluted with 0.3M, 0.5M, 1M KCL buffer B. Enriched RNA binding proteins migrated on 10% SDS-PAGE gel, and stain with coomassie blue and silver stain.

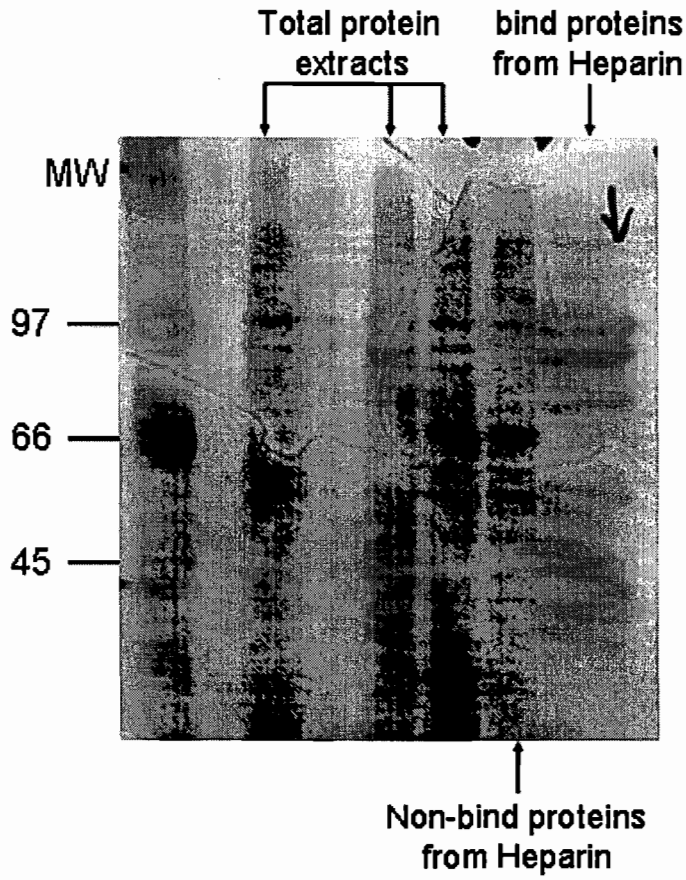

Fig 11. Total GC protein extracts were incubated with heparin-agarose beads, and eluted from by elution buffer (contain 0.5M KCL). Total protein extracts, non-binding proteins and heparin-binding proteins (20μg) were separated on SDS-PAGE gel, and stain with Coomassie blue.

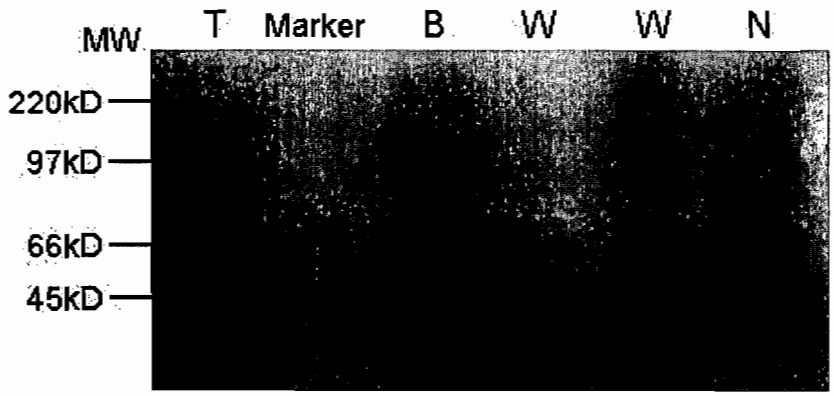

Fig 12. Total protein extracts (T) were incubated with Heparin-Agarose beads, and un-bound proteins (N) were removed from the beads, which were then washed 5 times, the supernatant from the last wash (W) was kept, and RNA binding protein (B) was finally eluted by high elution buffer (contain 0.5M KCL). All samples were examined by UV crosslinking, total protein extracts (T) and enriched RNA binding proteins (B) were both present at approximately 66kDa and 97kDa, no clear band was present in un-bound proteins (N) and the supernatant of the last wash (W).

	Activity of probe fractions (cpm)					
Biotin (%)	1 µl RNA	Un-bound RNA	Wash 1	Wash 2	Wash 3	Beads
0%	540 265	503 481	10 246	462	190	511
12%	481 351	307 642	22 350	10 756	9 990	88 940
25%	593 450	162 763	72 653	36 977	22 798	234 594
50%	439 264	105 875	66 091	32 386	51 981	165 046

Table 1. Binding activity of radioactive probes containing different amounts of biotin.

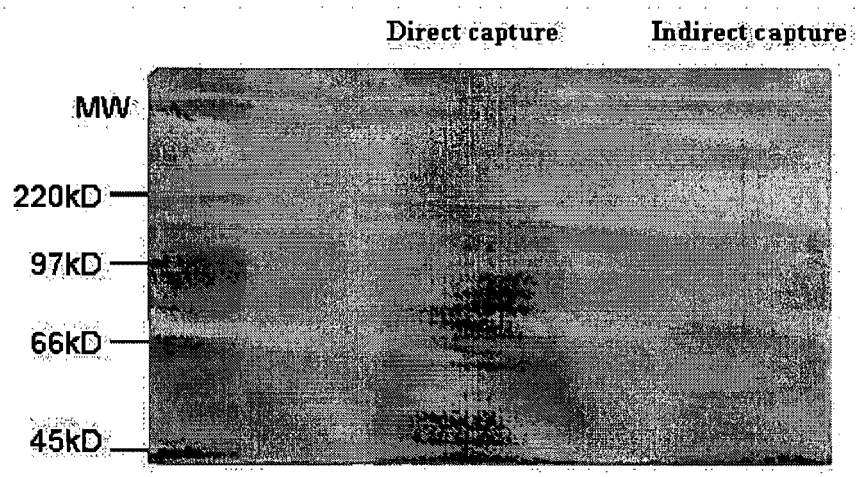

Fig 13. Enriched RNA binding proteins and biotinylated 3'UTR probes were used for isolation of the specific binding protein(s) by direct and indirect capture. P450arom 3'UTR binding protein(s) was eluted from streptavidin beads, and migrated on 10% SDS-PAGE gel, and silver-stained.

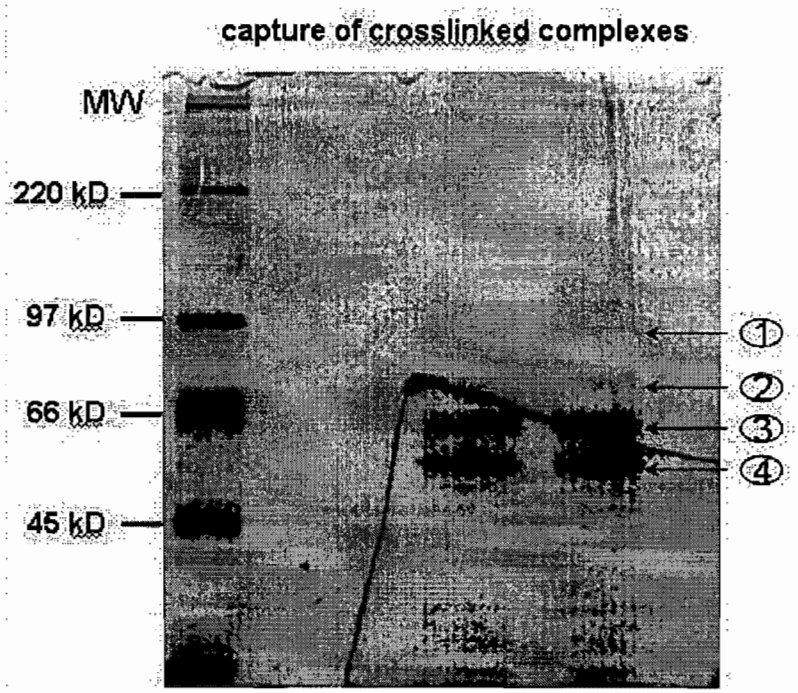

Fig 14. Pre-purified RNA binding protein (50μg) were incubated with 1000μg biotinylated P450arom 1/2 3'UTR RNA probes, after UV crosslinking, protein-RNA probe complex were incubated with streptavidin beads, P450arom RNA binding protein was eluted with high salt low pH elution buffer, and migrated on 10% SDS-PAGE gel, stained with silver.

CONCLUSION

Aromatase activity in bovine granulosa cells is dependent on continued protein synthesis (Sahmi M et al., 2006), and the mRNA encoding the bovine aromatase gene (Cyp19) is characterized by a long (3.5kb) 3'UTR and a short half-life. Further, stimulation of granulosa cells with different hormones alters the stability of the mRNA (Sahmi M et al., 2006). Therefore we proposed that posttranslational regulation of Cyp19 gene product may play an important physiological role in the ovary and perhaps in other estrogen-secreting tissues.

Previous studies in the laboratory (Sahmi, pervious studies) demonstrated that a section (+529 to +1714) of the 3'UTR encoding the Cyp19 gene binds to a protein(s) of approximately 70kDa, and this binding activity is observed in bovine granulosa cells, but not in corpus luteum, lung, liver, kidney, pancreas or bladder extracts. The objective of this project was to isolate this protein.

We tested multiple methods of affinity chromatography-based isolation, using the 3'UTR RNA as affinity matrix bound to solid support. Variable results were obtained. Elution of bound proteins did not produce sufficient amount of highly pure proteins for identification. Capture of crosslinked RNA-protein complex offered the most promising approach, but owing to the presence of RNA strands on eluted peptides, LC/LC-MS was not able to identify the proteins. Further work is required to identify the binding proteins.

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