INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI

A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor MI 48106-1346 USA
313/761-4700 800/521-0600

Université d'Ottawa · University of Ottawa

ROLE AND REGULATION OF PROSTAGLANDIN PRODUCTION IN GRANULOSA CELL DNA SYNTHESIS DURING OVARIAN FOLLICULAR DEVELOPMENT

By

Julang Li, M.Sc.

A thesis submitted to the School of Graduate Studies and Research, University of Ottawa in partial fulfilment of the requirement for the degree of Doctor of Philosophy,

Department of Cellular and Molecular Medicine, Faculty of Medicine

ACKNOWLEDGEMENTS

I wish to express my thanks to the following individuals for their support during the past four years in my Ph.D. studies:

Dr. Benjamin K. Tsang, my supervisor. His enthusiasm in science and research, extensive experience in Reproductive Biology and strict adherence to scientific criteria have been a constant source of inspiration. His understanding and confidence in my work have always been an encouragement and support when the going was hard. I really treasure my learning experience in his laboratory, which has provided the stimulating and supportive environment for the full development of my research potential.

Drs. William Gibb and Gordon Kinson, members of my Advisory Committee for their scientific guidance and advice.

Dr. Anthony Krantis, Director of Graduate Studies of the Department of Physiology, who has always provided immediate support when I needed it.

Professor Ming Li, my teacher and colleague, who introduced me to Canada and to the field of Reproductive Biology. His support has been instrumental in my career development for the past 15 years. I thank him for the opportunity in research collaboration which resulted in three publications.

Dr. Martine Lafrance who taught me the techniques of RIA and zymography and Dr. William Yan who guided me in my first venture into molecular biology.

Mr. Jon Soboloff for introducing me to the graphic programs and basketball team. I enjoyed the many discussions we shared, ranging from research to different cultures. Mr. David Boone for his helpful discussion and encouragement. Drs. Eli Karakji, Jong-Min Kim

and Qiang Feng, Mr. Raman Manchanda and Mrs. Danielle Schneiderman and others in the laboratory for their help and memorable time we shared together.

Mrs. Marguérite Boone for her kind help in brushing up the English of the thesis.

Mrs. Alana Summers, Donna Mulder and Terry VanGulik; they have always been nice and helpful.

My parents, aunts and brothers; their trust and support have been a constant source of energy that enable me to move forward.

My husband Yuehua Cai and our daughter Kimberly Cai for their love, understanding and sacrifices which have made my dream come true.

ABSTRACT

Although much is known concerning the production and action of growth factors in the ovary, the present knowledge on their mechanisms of action remains incomplete. The aim of this study was to examine the role and regulation of eicosanoids in the mitogenic actions of TGFα on granulosa cells during follicular development, as well as its involvement in the regulation of urokinase plasminogen activator, an enzyme playing an important role in tissue remodelling.

Granulosa cells from the first (F1), fifth and sixth (F5-6) largest preovulatory follicles were cultured for up to 24 h in the presence of TGFα (0.1-10 ng/ml) and/or TGFβ (4-20 ng/ml) or TGFα together with inhibitors of phospholipase A_2, cyclooxygenase or lipoxygenase, leukotrienes (LTs), lysophosphatidyl choline (LPC), lysophosphatidic acid (LPA) and/or prostaglandins (PGs). The release of PG into the incubation medium was determined by RIA, and DNA synthesis was assayed by measurement of [^{3}H]thymidine incorporation into DNA. TGFα stimulated PG secretion in a concentration-dependent manner. This stimulation was suppressed concentration-dependently by hydroxyurea (1.5-6 mM), a DNA synthesis inhibitor. TGFα-induced DNA synthesis in F1 and F5-6 granulosa cells was suppressed by inhibitors of phospholipase A_2 (ONO-RS-82, 0.1 - 5.0 μM; aristolochic acid, 5-25 μM; 7,7-dimethyleicosadienoic acid, 5 -100 μM) and cyclooxygenase (ibuprofen, 15 - 60 μM; naproxen, 37.5 - 150 μM), while an inhibitor of lipoxygenase (MK-886, 0.1 to 2 μM) was ineffective. The inhibition was concentration-dependent and could be attenuated by exogenous PGE_2 (0.5-50 μM). Likewise, PGF and PGE production was suppressed by ONO-RS-82, ibuprofen, naproxen, and indomethacin. Moreover, PGE_2 and,

to a lesser extent, $PGF_{2\alpha}$ (0.5 - 50 μM) increased basal [^{3}H]thymidine incorporation and enhanced DNA synthesis induced by a submaximal stimulatory concentration of TGFα (1 ng/ml). The mitogenic effect of PGs was more evident in granulosa cells from F5-6 than from the F1 follicle. In contrast, leukotrienes (LTB_4, LTC_4, LTD_4 : 20-1000 ng/ml), (+)5(s)-hydroxy-(6E,8Z,11Z,14Z)-eicosatetraenoic acid (5-HETE, 0.2-4 μM) and lysophospholipids (LPA and LPC, 0.1-10 μM) had no effect on granulosa cell DNA synthesis, irrespective of the stage of ovarian follicular development and the presence of TGFα and PGE_2. The stimulation of PG secretion by TGFα was maximal in F3 and F1 granulosa cells. TGFß suppressed both basal and TGFα-stimulated PG secretion and the inhibition appeared to be more pronounced in the less differentiated cells. These findings are consistent with our concept that an increase in PG production is an important element in the TGFα regulated cascade of biochemical events leading to chicken granulosa cell mitogenesis during hen ovarian follicular development.

To investigate the role of cyclooxygenase (COX) and cytosolic phospholipase A_2 ($cPLA_2$) in the regulation of hen granulosa cell PG production by TGFα and TGFß, COX and $cPLA_2$ protein and mRNA levels were also determined. The increase in PG secretion produced by TGFα was accompanied by an elevation of COX II content which was concentration- and time-dependent; the maximum response was observed within 6-12h. Maximal increase in COX II mRNA abundance was evident at 4 and 8 h in cells from F1 and F5-6, respectively. While TGFα-stimulated PG secretion was higher in cells from a mature follicle (F1), the magnitude of change in COX II mRNA abundance and protein content was, however, not dependent on follicular maturation. TGFß significantly suppressed basal and

TGFα-induced COX II transcript levels. COX I transcript, however, was undetectable irrespective of the presence of TGFα, duration of culture or follicular maturation. Treatment with TGFα caused a shift from an electrophoretically fast migrating protein to a slow migrating form, a phenomenon sensitive to inhibitors of serine /threonine kinase as well as MAP kinase pathways, suggesting phosphorylation and activation of $cPLA_2$ may possibly be involved in the action of the growth factor. In contrast, TGFβ suppressed $cPLA_2$ expression, as evident by a marked decrease of $cPLA_2$ mRNA abundance and protein content, but failed to prevent the mobility shift of $cPLA_2$ induced by TGFα. Consistent with the influence of this growth factor on PG production previously observed and in contrast to the action of TGFα, the inhibition of $cPLA_2$ by TGFβ is more pronounced in granulosa cells at the early stage of follicular development. These results demonstrate the regulation of granulosa cell COX II and $cPLA_2$ at the transcriptional and post-transcriptional levels during ovarian follicular development. In addition, it is proposed that the activation by TGFα and down-regulation by TGFβ of $cPLA_2$ may be important regulatory mechanisms in the control of granulosa cell PG production and thereby the mitogenic response of the cells to the growth factors during ovarian follicular development.

Although Mothers Against dpp (MAD) and its related proteins (MADR) are believed to be important components of the cell signalling pathway for the transforming growth factor beta (TGFβ) superfamily, the presence and regulation of these signalling molecules in ovarian cells by TGFβ is not known. We have examined the presence of MADR2 and MADR1, two members of the MADR family, in granulosa cells at different stages of follicular development,

and the influence of TGFβ *in vitro* on their expression, particularly in the context of TGFβ-induced down regulation of $cPLA_2$. We have demonstrated the presence of MADR2 and MADR1 in hen granulosa cells at different stages of follicular development. The expression of MADR2, but not of MADR1, was up-regulated by TGFβ *in vitro* in a concentration- and time-dependent manner. Granulosa cell MADR2 expression was maximal during early stages of follicular development when the granulosa cell $cPLA_2$ system is most responsive to the growth factor. The changes in MADR2 expression were accompanied by reciprocal alterations in the expression of $cPLA_2$. These findings are consistent with the hypothesis that homologous up-regulation of MADR2 in the granulosa cell may be an important determinant in its follicular stage-specific responsiveness to TGFβ and possibly in the suppression of $cPLA_2$ gene transcription by the growth factor.

To investigate whether TGFα-induced granulosa cell proliferation is accompanied by activation of the tissue remodelling process, the response of plasminogen activator (PA) expression and activity to TGFα were also determined. PA activity was stimulated by TGFα. Both basal and the growth factor-induced PA activity was greater in granulosa cells from early stage of follicular development (F5-6) when granulosa cells were most proliferatively active. The regulation of PA by TGFα was also at the level of protein and mRNA expression. Indomethacin inhibited TGFα-induced PG secretion but had no significant effect on PA activity, suggesting that the regulation of PA activity in granulosa cells by the growth factor is independent of PG secretion and action during preovulatory follicular maturation.

These findings suggested that, in addition to the well established role of

gonadotrophins in the regulation of ovarian follicular development, transforming growth factor α and β are important intrafollicular regulators of granulosa cell mitogenesis. Moreover, PG synthesis and action may mediate the regulation of DNA synthesis by these growth factors, a process coupled with the expression of urokinase PA believed to be involved in ovarian tissue remodelling during folliculogenesis.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
cAMP	Cyclic 3',5' adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
COX	Cyclooxygenase
$cPLA_2$	Cytosolic phospholipase A_2
dCTP	deoxy cytidine triphosphate
DNA	Deoxyribonucleic acid
DEDA	7,7-Dimethyleicosadienoic acid
DEPC	Diethylpyrocarbonate
ECL	Enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
FSH	Follicle stimulating hormone
GH	Growth hormone
IGF	Insulin-like growth factor
IL-1	Interleukin-1
Kb	Kilobase
K_d	Dissociation constant
Kda	Kilodalton
LH	Luteinizing hormone
LWF	Large white follicle

LT	Leukotriene
M199	Medium 199
MAD	Mothers against dpp
MADR	Mothers against dpp related protein
MEM	Minimum essential medium
mRNA	Messenger ribonucleic acid
PBS	Phosphate buffered saline
PBSG	Phosphate buffered saline with gelatine
PA	Plasminogen activator
PAc	Cell-associated plasminogen activator
PAs	Secreted plasminogen activator
PAI-1	Plasminogen activator inhibitor-1
PAGE	Polyacrylamide gel electrophoresis
PGE	Prostaglandin E
$PGF_{2\alpha}$	Prostaglandin F2α
PLA_2	Phospholipase A_2
PMSF	Phenylmethylsulfonyl fluoride
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulfate
SEM	Standard error of mean
$sPLA_2$	Secreted phospholipase A_2
SYF	Small yellow follicle

tPA	Tissue-type plasminogen activator
TBS	Tris buffered saline
[^{3}H]	Tritium
TGFα	Transforming growth factor alpha
TGFβ	Transforming growth factor beta
TNFα	Tumor necrosis factor alpha
uPA	Urokinase-type plasminogen activator
uPAR	Urokinase receptor

LIST OF FIGURES

LIST OF TABLES

I. LITERATURE REVIEW

A. General Structure of Avian Ovary

The reproductive system of the egg laying hen consists of a single left ovary and its oviduct. The left ovary is attached to the cephalic end of the left kidney by the mesovaria ligament. The ovary of the immature birds consists of a mass of small ova, at least 2,000 of which are visible to the naked eye. Only relatively few of these (200-500) reach maturity and are ovulated within the lifespan of most domestic species, and considerably fewer mature in wild ones. The functionally mature ovary of the hen (Fig. 1) is commonly arranged with a hierarchy of six large yolk-filled follicles (2-4 cm in diameter), accompanied by a greater number of yellow follicles [termed small yellow follicle (SYF); 7-15 mm diameter] and of white follicles [termed large white follicles (LWF); 2-7mm diameter]. The hierarchical follicles are committed to ovulate 24h from each other and, according to their relative size, are designated as F1, F2, F3, F4, F5 and F6. Within the hierarchy, F1 is the first follicle to ovulate. The follicular wall consists of concentric layers of tissue which surround the oocyte and yolk, including: (i) the oocyte plasma membrane, (ii) the perivitelline membrane, (iii) granulosa cells, (iv) basal lamina, and (v) the theca, which consists of the interna and externa layers (Fig.2).

Figure 1: **Follicular hierarchy of the egg-laying hen**

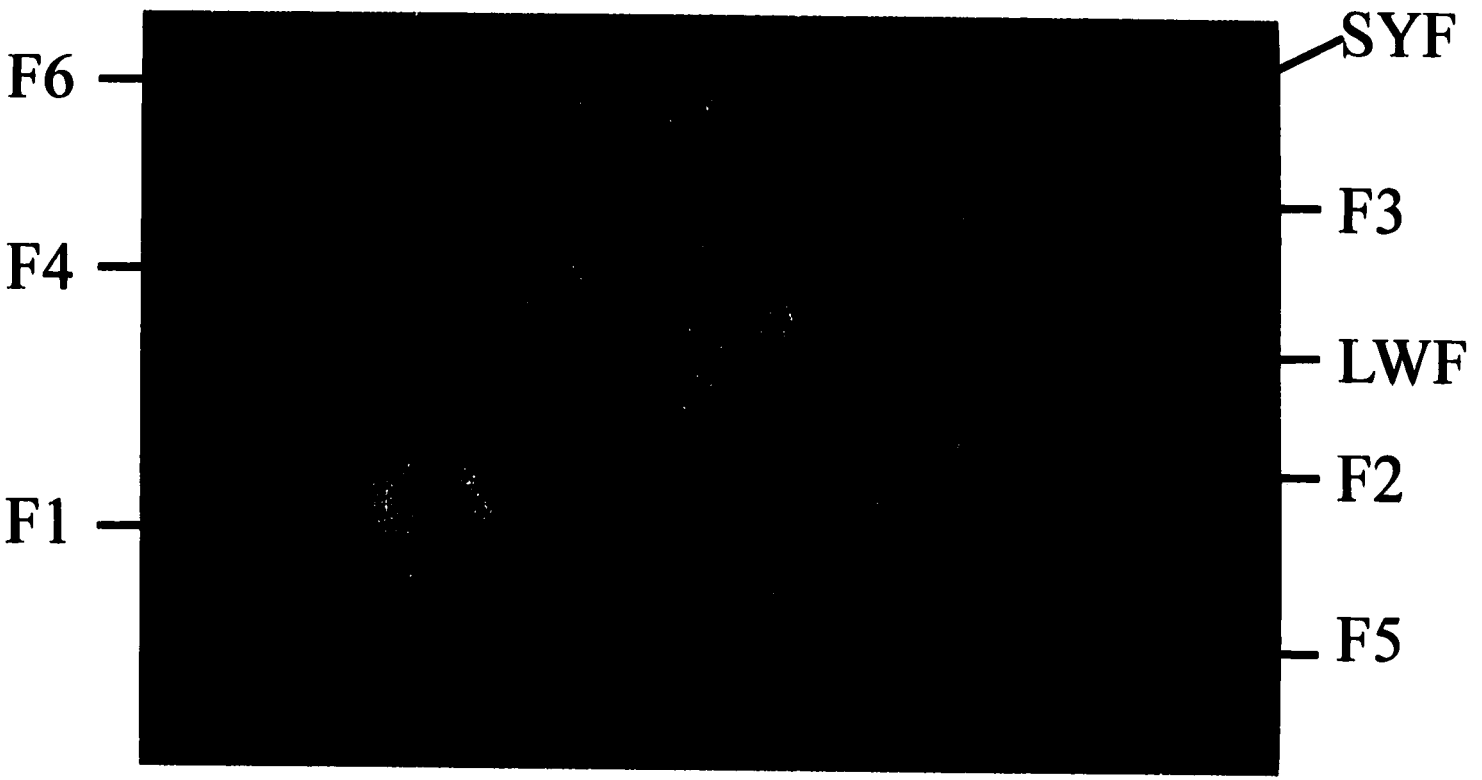
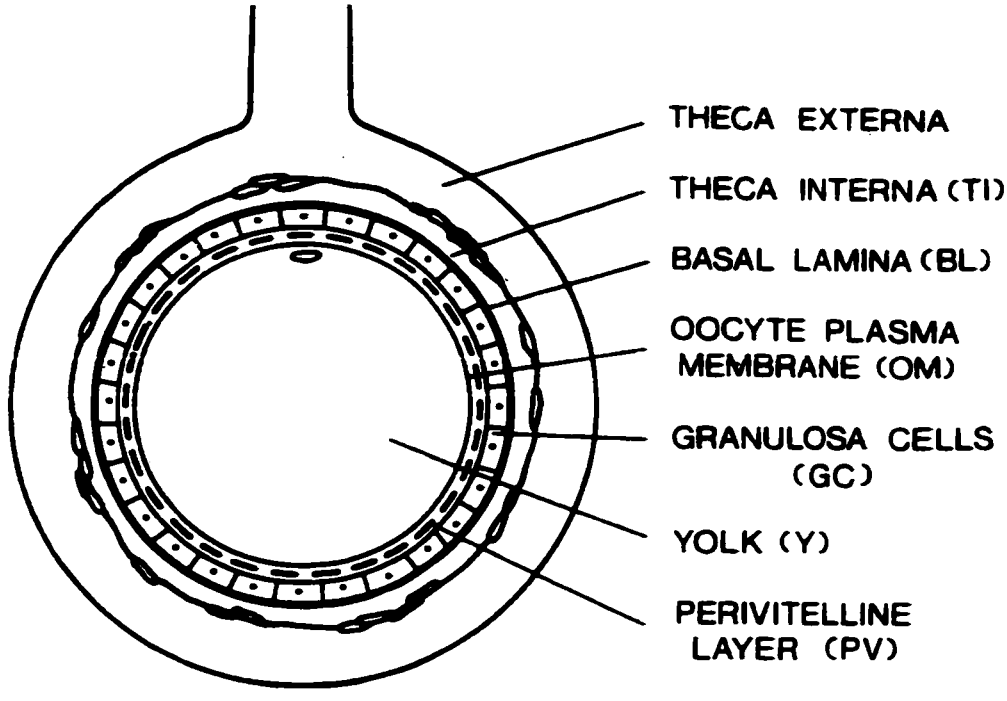

F6
F4
F1
SYF
F3
LWF
F2
F5

Figure 2: Diagram showing a cross-section of a hen follicle (from Gilbert, 1979).


THECA EXTERNA
THECA INTERNA (TI)
BASAL LAMINA (BL)
OOCYTE PLASMA MEMBRANE (OM)
GRANULOSA CELLS (GC)
YOLK (Y)
PERIVITELLINE LAYER (PV)

B. Follicular Development

Ovarian follicular development is dependent on the proliferation and differentiation of granulosa and theca cells which are regulated by gonadotropins and intra-ovarian factors including growth factors, cytokines and prostaglandins (Wang et al., 1992; Khan et al., 1980; Takakura et al., 1989; Galway et al., 1989; Lafrance et al., 1993a, b; Li and Tsang, 1995). Growth of the avian follicle can be divided into three phases: (i) slow growth phase of follicles (0.05-1 mm in diameter) which may last from months to years, (ii) a 2-month period of increasingly rapid growth during which the yolk protein is deposited, and (iii) a rapid growth phase in the final seven to eleven days before ovulation when the follicle increases in size (from 8 mm to 37 mm in diameter) and weight (from 0.08-1.5 g to 18 g; Marza and Marza, 1935).

C. Follicular Atresia

Two major stages of cell degeneration can be distinguished in the mammalian ovary: degeneration of germ cells (attrition), which accounts for the largest loss of oocytes (Beaumont and Mandl, 1962) and occurs mainly prenatally, and follicle degeneration (atresia), which occurs during postnatal reproductive life. In the human ovary, two million oocytes are found at birth, but only approximately 400,000 follicles are present at the onset of puberty. Moreover, only about 400 follicles are ovulated during the female reproductive life (Baker, 1963). Thus, only a small fraction of the potential pool of oocytes will ever ovulate and the majority will undergo demise.

Ovarian follicular atresia is a hormonally controlled and genetically programmed process, whereby degenerating follicles are eliminated in a coordinated fashion (Hsueh et al., 1994). Using internucleosomal DNA fragmentation as a marker, follicle atresia in

diverse species ranging from avian to human has been shown to result from apoptotic cell death (Hughes and Gorospe 1991; Tilly et al., 1991; Quirk et al., 1995). In mammals, atresia occurs at all stages of follicular development. Sufficient FSH support is the most critical stimulus for the antral follicles to escape atresia and reach the preovulatory stage of development (Chun et al., 1996; Hirshfield and Midgley, 1978). Interestingly, in the avian ovaries, the susceptibility of granulosa cells to undergo apoptosis is dependent on the stage of follicular development. A higher rate of atresia occurs in follicles not selected into the preovulatory hierarchy (LWF; Johnson et al., 1996).

D. Granulosa Cell

1. Proliferation of Granulosa Cells

In mammals, the first sign of growth is the resumption of proliferation of the squamous granulosa cells, which can be demonstrated by autoradiography following [^{3}H]thymidine incorporation (Hirshfield, 1991). The time required for each follicle to grow from one stage to another is dependent on the rate of granulosa cell proliferation which may vary substantially. During hen ovarian follicular development, the rate of granulosa cell proliferation is highest in small developing follicles (Lafrance et al., 1993b).

Signals from one follicular compartment may influence cell proliferation via paracrine mechanisms. For example, the maturing oocyte is known to regulate granulosa cell proliferation during the early stages of follicular growth (Satoh et al., 1985). Theca cells appear to secrete factors that influence the rate of granulosa cell proliferation. Co-culture of granulosa cells with theca cells, or culture of granulosa cells in theca-conditioned medium increases granulosa cell numbers and [^{3}H]thymidine incorporation (Bendell et al., 1988). In addition, the microvascular network of maturing follicles is at least two times as

dense as that around less mature follicles (Zeleznik and Hillier, 1984), and differences in blood flow are expected to alter nutrient availability to proliferating cells, which in turn influences their generation times (Hirshfield, 1991).

2. Granulosa Cell Differentiation

Granulosa cell differentiation is indicated by increased gonadotropin receptor expression and steroidogenesis. In mammals, FSH receptors are the only gonadotropic receptors expressed on granulosa cells of preantral follicles, and are present on granulosa cells when the oocyte ceases to grow, although in a masked non-functional form (Richards and Midgley, 1976; Uilenbroek and Richards, 1979). The conversion of the receptor to functional membrane-bound protein is induced by the presence of the hormone (Ford and LaBarbera, 1988). FSH- receptor interaction causes an increase in FSH receptor number in the antral follicles (Uilenbroek and Richards, 1979). Continuous exposure of these follicles to the gonadotropin down-regulates FSH receptors on granulosa cells of preovulatory follicles (LaPolt et al., 1992) but increases binding of [^{125}I] hCG to granulosa cells *in vivo*, which indicates that FSH is capable of inducing granulosa cell hCG/LH receptors (Magoffin and Erickson, 1982). Induction of LH receptors in granulosa cells has been confirmed *in vitro* (Piquette et al., 1991) and *in vivo* (Segaloff et al., 1990). Withdrawal of FSH results in the loss of both FSH and LH receptors and death of granulosa cells (Richards, 1980; Erickson, 1986). Interestingly, the number of hen granulosa cell FSH receptor decrease but those of LH receptor increase with follicular maturation (Ritzhaupt and Bahr, 1987, Johnson et al., 1996).

Three major classes of steroid are produced by follicular cells: progestins, androgens, and estrogens. The precursor of all ovarian steroids is cholesterol, a 27-carbon

molecule, which is composed of a steroid nucleus (three cyclohexane rings and a cyclopentane ring), two methyl groups and an eight carbon side chain. Cleavage of the side chain at 20,22-carbon leads to the formation of a 21-carbon steroid, progesterone. Further cleavage of the side chain produces 19-carbon steroids (androgens). Removal of the C-19 methyl group forms 18-carbon steroids (estrogens). The production of these steroids is dependent on the induction and/or activation of key steroidogenic enzymes present in both the theca and granulosa cells. A number of key steroidogenic enzymes are lacking or weakly expressed in the granulosa cells of follicles at early stages of follicular development. In the presence of FSH, the granulosa cells acquire aromatase activity (for the conversion of androgen to estrogen) *in vivo* and *in vitro* (Zeleznik et al., 1974; Erickson and Hsueh, 1978). Both FSH and LH stimulate granulosa cell progesterone secretion (Hsueh et al., 1984) by inducing the activity of 3β-hydroxysteroid dehydrogenase (3β-HSD) involved in the conversion of pregnenolone to progesterone. Moreover, LH stimulates the production of aromatizable androgens and progesterone by the theca cells (Hsueh et al., 1984). Interestingly, in contrast to mammals, androgens in hen ovarian follicles are produced by the granulosa and theca cells while estrogens are produced by the theca cells. In addition, the oocyte is also known to regulate granulosa cell steroidogenesis (Vanderhyden and Tonary, 1995; Vanderhyden et al., 1993). The expression of enzymes involved in estrogen and progesterone synthesis and secretion are often used as functional indices of granulosa cell differentiation.

3. Granulosa Cell Apoptosis

Follicular atresia occurs via apoptosis, and deoxyribonuclease I, a Ca^{++}/ Mg^{++} - dependent endonuclease is believed to be responsible for apoptotic DNA fragmentation

detected in granulosa cell death (Boone et al., 1995; Boone and Tsang, 1997). It has been hypothesized that apoptosis is responsible for the demise of the granulosa cells and ultimately that of the follicle (Zeleznik et al., 1989). This hypothesis is supported by ultrastructural and morphological studies of granulosa cells identifying characteristics of apoptosis, including membrane blebbing, chromatin condensation and cellular fragmentation and formation of apoptotic bodies (Hay et al., 1976). Moreover, the DNA degradation pattern of apoptosis has been observed in granulosa cells but not theca cells from atretic follicles, and is absent in follicles undergoing rapid growth and development (Hughes and Gorospe, 1991). Granulosa cell apoptosis is regulated by steroid hormones, gonadotropins, gonadotropin releasing hormone (GnRH) and growth factors (Tilly et al., 1992a; Billig et al., 1993, 1994), which suggests that apoptosis is associated with the process of follicular atresia and granulosa cells may be the major cells type involved.

E. Regulation of Granulosa Cell Function

1. Pituitary Hormones

FSH acts as a permissive signal of granulosa cell maturation; it is the granulosa cells, rather than the FSH that determine the nature of the cellular response (Hirshfield, 1991). All follicles in the ovary are probably exposed equally to FSH, but only follicles in the late stage of clonal expansion respond to the hormone by acquiring features of functional maturation. The nature of the response has already been predetermined (Gurdon, 1987) and the FSH stimulus is simply needed to trigger its expression. After exposure to FSH, the follicle acquires the enzymatic machinery needed for the end stage maturation (luteinization). Exposure to LH promotes the follicle into completion of the preprogrammed developmental process that was set in place during the initial stages of

folliculogenesis, weeks or months earlier (Hirshfield, 1991).

Both FSH and LH stimulate granulosa cell progesterone secretion (Hsueh et al., 1984). LH stimulates the production of aromatizable androgens and progesterone by the theca cells (Hsueh et al., 1984). In addition to their roles in steroid production, gonadotropins are also involved in the regulation of extracellular matrix production and tissue remodelling. FSH suppresses fibronectin secretion (Skinner and Dorrington, 1984; Skinner et al., 1985) but stimulates proteoglycan biosynthesis by granulosa cells (Salustri et al., 1990). In hen granulosa cells, fibronectin production increases with follicular maturation and is induced by LH (Asem et al., 1992). Granulosa cell PA activity is altered by FSH (Karakji and Tsang, 1995a&b).

2. Cytokines

Although the cytokine tumour necrosis factor alpha (TNFα) was initially thought to be tumour-selective, it has become clear that certain non-tumour cells also possess TNFα receptors and that this cytokine regulates non-cytotoxic activities in these cells (Adashi et al., 1989). Although TNFα may be produced locally by resident ovarian macrophages (Adashi et al., 1989), immunohistochemical and *in situ* hybridization studies have demonstrated that follicle cells (oocyte, granulosa and theca) are also sources of TNFα (Roby and Terranova, 1989; Chen et al., 1993; Marcinkiewicz et al., 1994). TNFα is capable of attenuating granulosa cell differentiation *in vitro* (Adashi et al., 1989; Darbon et al., 1989). While this cytokine inhibits FSH-stimulated aromatase activity and progesterone production in cultured granulosa cells (Emoto and Baird, 1988; Darbon et al., 1989, Adashi et al., 1990), it stimulates granulosa-lutein cell proliferation (Wang et al., 1992). TNFα increases progesterone production by preovulatory follicles and prostaglandin $F_2\alpha$ synthesis

in granulosa cells (Roby and Terranova, 1988; Zolti et al., 1990).

At the ovarian level, a significant amount of interleukin-1 (IL-1)-like activity has been detected in follicular fluid (Khan et al., 1980; Takakura et al., 1989). IL-1β is produced by resident rat ovarian macrophages which are present at various stages of folliculogenesis (Adashi, 1992a, b). In addition, IL-1β and its receptor have been localized in theca-interstitial and granulosa cells (Simon et al., 1994), suggesting local synthesis and actions of this cytokine. IL-1β inhibits the FSH induction of LH receptors in granulosa cells (Gottshall et al., 1988a). Although the cytokine suppresses gonadotropin-induced follicular steroidogenesis (Gottshall et al., 1987, 1988b; Hurwitz et al., 1991), it has a growth promoting effect on cultured rat granulosa cells (Fukuoka et al., 1992). IL-1β stimulates PGE and $PGF_{2\alpha}$ release from preovulatory follicles and enhances LH-induced ovulation in perfused ovaries (Brannstrom et al., 1993; Takehara et al., 1994).

3. Growth Factors

a. Epidermal growth factor and transforming growth factor α

Epidermal growth factor (EGF) and transforming growth factor alpha (TGFα) are structurally related but have distinct single chain polypeptides of 53- and 50- amino acids, respectively (reviewed by May & Schomberg, 1989). Although encoded by different genes, these growth factors bind to the same receptor (Todaro et al., 1980). EGF is a principal regulator of proliferation, differentiation and pattern formation in a variety of tissues and cell types (reviewed by Carpenter & Cohen, 1990). This 6 kDa polypeptide is produced by the cleavage of an EGF precursor, a large 1168-residue transmembrane protein. EGF and TGFα production in the ovary are regulated by gonadotropins (Roy and Greenwald, 1990; Kudlow et al., 1987) and receptors for EGF (and therefore for TGFα) are known to be

present in granulosa cells (Vlodavsky et al., 1978; Jones et al., 1982; Buck and Schomberg, 1988). Both EGF and TGFα have been shown to stimulate PA activity in cultures of granulosa cells (Galway et al., 1989; Lafrance et al., 1993a,b). Depending upon prior hormonal stimulation and the presence of other regulatory factors in the culture medium, EGF and TGFα can stimulate or inhibit proliferation or differentiation of granulosa cells *in vitro* (Leal et al., 1990). The proliferative activity of granulosa cells from many species including hen and human (but not rat) is induced by EGF or TGFα (Gospodarowicz and Bialecki, 1979; Lafrance et al., 1993a). TGFα, like EGF, is a potent inhibitor of gonadotropin-induced granulosa cell differentiation (May and Schomberg, 1989; Schomberg et al., 1983). Both EGF and TGFα have been shown to inhibit estrogen and progesterone synthesis and LH receptor expression in rodent granulosa cells (Hsueh et al., 1984; Mondschein and Schomberg, 1981). Similarly, LH induced hen granulosa cell progesterone production is suppressed by EGF and TGFα (Tilly and Johnson 1990, Peddie et al., 1994).

The EGF receptor is a single polypeptide chain (Mohammadi, 1993). It is monomeric and inactive in the absence of the growth factor. Binding of EGF to the extracellular domain causes receptor dimerization and autophosphorylation . Autophosphorylation enhances the capacity of the receptor to phosphorylate intracellular effectors. Phosphorylated tyrosines on the activated EGF receptor are recognized by conserved sequences on target proteins called SH2 domains (Schlessinger and Ullrich, 1992). The signal transduction pathways activated by EGF include the phosphatidylinositol pathway, which leads to activation of protein kinase C and an increase in intracellular Ca^{++} concentration, and the Ras pathway, which leads to MAP kinase activation (Boonstra et al., 1995).

b. Transforming growth factor β

The transforming growth factor β (TGFβ) superfamily comprises a large group of growth and differentiation factors. They have been identified in a wide variety of species (from insects to mammals), and have been shown to have distinct but diverse growth and differentiative functions in many physiological systems. Among the most studied members of this superfamily are TGFβs, activins, and bone morphogenetic proteins (BMPs). These polypeptides have been reported to mediate numerous physiological processes including cell differentiation, tissue repair, bone formation, regulation of hormone secretion, immune response and various developmental functions in many organisms (Massague et al., 1996a; Gaddy-Kurten et al., 1995). For example, the decapentaplegic (*dpp*) product in *Drosophila*, a BMP homologue, is believed to play a role in the determination of cell fate during development (Padgett et al., 1987; Ferguson and Anderson, 1992). In *Xenopus*, BMP mediates ventralization of mesoderm. Timely removal of this signal is required for neural induction (Smith et al., 1995; Hawley et al., 1995; Wilson and Hemmati-Brivanlou, 1995). On the other hand, activin induces formation of dorsal mesoderm (Smith et al., 1995). TGFβ1is important in cell growth, differenciation and apoptosis of many cell type (Van Obberghen-Schilling et al., 1988, Lin & Chou 1992). TGFβ1 homozygous mutant animals exhibited a multifocal inflammatory dicease and the offspring were less than expected, suggesting the occurrence of some type of prenatal lethality (Shull & Doetschman 1994). In the ovary, TGFβ stimulates FSH-induced aromatase activity, enhances FSH-dependent progesterone production, augments FSH-induced granulosa cell LH receptor expression and induces inhibin biosynthesis in granulosa cell cultures (Skinner et al., 1987; Adashi and Resnick, 1986; Knecht et al., 1987; Zhang et al., 1988). Thus, it is possible that TGFβ

serves as an important paracrine/autocrine intra-follicular factor (Hernandez et al., 1987; Mulheron and Schomberg, 1990).

Members of the TGFβ superfamily exert their biological effects by interacting with two types of transmembrane receptors (type I and type II) with serine and threonine kinase activity (Massague et al., 1996b; Gaddy-Kurten et al., 1995; Mathews and Vale, 1991). These receptors comprise a short, cysteine-rich extracellular domain and an intracellular domain with specificity toward serine and threonine residues. The type II receptors of TGFβ and activin are involved in initial ligand binding. The ligand-bound type II receptors recognize and recruit type I receptors to form a heteromeric complex. In this complex, the type I receptors are transphosphorylated by the kinase domain of the type II receptors, eliciting the propagation of downstream signals (Wrana et al., 1994; Attisano and Wrana, 1996). The ligand-receptor interaction is different for BMP receptors in that either the type I or type II receptors are capable of low affinity interaction with the BMP ligands. However, both receptors together enable high affinity binding with the ligand, and the formation of the heteromeric ligand-receptor complexes is required for BMP signalling.

More recently, the novel gene family MAD and its related proteins (MADR) have been identified in a variety of species as important components of the signal transduction pathway involving serine/threonine kinase receptor signalling, including that of TGFβ (Graff et al., 1996; Hoodless et al., 1996; Liu et al., 1996; Newfeld et al.,1996; Sekelsky et al., 1995; Thomsen, 1996; Wiersdroff V et al., 1996). As shown in Table 1, at least five MADR proteins have so far been characterized in vertebrates. Smad1 (MADR1) and Smad2 (MADR2) have been shown to mimic the effects of BMP and activin, respectively, both in Xenopus and in mammalian cells (Liu et al., 1996; Graff et al., 1996; Eppert et al., 1996).

MADR2, Smad3 (MADR3, a close homologue of MADR2) and the related protein DPC4 (MADR4, a tumour-suppressor gene product) mediate TGFβ actions (Zhang et al., 1996; Eppert et al., 1996). After ligand-receptor binding, MADR1 phosphorylation is specifically induced by BMP2 (Hoodless et al., 1996). Neither TGFβ nor activin can induce MADR1 phosphorylation. In contrast, MADR2 is phosphorylated specifically in response to TGFβ but not to BMP2 (Eppert et al., 1996). It has been demonstrated that, in certain regions of the embryo, MADR2 is responding to a signal which alters its subcellular localization and presumably leads to changes in cell fate (Baker and Harland, 1996). It has been suggested that activin receptor activation results in the binding of MADR2 , but not of MADR1, to FAST-1 to form a site-specific transcriptional regulatory complex, which has an important role at the promoter region of an activin-responsive gene (Chen et al., 1996a & b). Although these studies suggest that MADRs may act as transcriptional regulators, further investigations are required to identify ligand-regulated physiological target expression mediated by these MADR proteins. In addition, the presence and regulation of MADR2 in the ovary by TGFβ have not been examined.

Table 1: TGFβ Super-family and Mother Against Dpp Related Proteins (MADRs)

Family Members	MADR Proteins
Bone Morphogenetic Proteins (BMPs)	MADR1, MADR5, DPC4
Transforming Growth Factor Beta (TGFβ)	MADR2, MADR3, DPC4
Activin	MADR2, MADR3, DPC4

F. Eicosanoids

1. Eicosanoid biosynthetic pathways

Prostaglandins, thromboxanes, and leukotrienes are derived from essential fatty acids and constitute a unique class of polyunsaturated, hydroxylated 20-carbon fatty acids. Phylogenetically, these compounds are old and evoke a variety of biological actions at low concentrations in diverse tissues and organs. Prostaglandins are produced in a wide variety of tissues and the production of specific subtypes seems to be tissue specific. In many cases, these compounds appear to act as local paracrine modulators of cellular function. They are metabolized rapidly and have a short duration of biological action (Behrman & Romero,1991). Since the enzymes that catalyze the formation of prostaglandins, leukotrienes and thromboxanes exist in a substrate-limiting environment, liberation of arachidonic acid from esterified stores results in prompt biosynthesis of these products (Behrman & Romero,1991).

a. Phospholipase A_2

Phospholipase A_2 (PLA_2) is a family of enzymes that catalyze the hydrolysis of the sn-2-ester bond of phospholipids. This reaction can result in arachidonic acid (AA) formation, the rate limiting step in the biosynthesis of arachidonic acid metabolites such as prostaglandin and leukotrienes. The Ca^{++}- dependent PLA_2 family can be divided into the low molecular weight secreted form ($sPLA_2$) and the high molecular weight cytosolic form ($cPLA_2$).

Based on its primary structure, $sPLA_2$ can be divided into two groups, pancreatic group I (PLA_2-I) and arthritic group II (PLA_2-II, Heinrikson et al., 1977). PLA_2-I is mainly synthesized in the pancreas as an inactive zymogen, which is further converted into the

active enzyme by proteases, and has been thought to serve as a digestive enzyme (de Haas et al., 1968). However, recent findings indicating the presence of PLA_2-I in nondigestive organs such as lung and spleen (Sakata et al., 1989; Tojo et al., 1988) suggest that the role of this enzyme is not limited to digestive processes, but may also have other distinctive physiological functions. In fact, it has been reported that PLA_2-I is involved in several biological responses, including stimulation of DNA synthesis and prostaglandin E_2 production (Kishino et al., 1992; Tohkin et al., 1993). PLA_2-II (14 kDa) is found in several cells and extracellularly when released in response to proinflammatory mediators such as IL-1β or tumor necrosis factors (TNFs). Its presence in inflammatory fluids, tissue exudates or serum has implicated PLA_2-II in inflammation (Vadas et al., 1985; Seilhamer et al., 1989).

Cytosolic PLA_2 is a novel member of the PLA_2 family and is distinct from $sPLA_2$ in a number of key characteristics: (i) it selectively cleaves phospholipid-containing arachidonic acid at the sn-2 position, whereas $sPLA_2$ is not AA specific but prefers ethanolamine-containing phosphoacylglycerols, (ii) it is activated by physiological concentrations of Ca^{++} (nM), while activation of $sPLA_2$ requires micromolar concentrations of the cation, and (iii) its activity is regulated by phosphorylation while $sPLA_2$ lacks phosphorylation sites (Bauldry et al., 1996). The charateristics of the PLA_2 family are summarized in Table 2.

Table 2: Characteristics of Phospholipase A_2 (PLA_2)

Type	Localization	Activation by Phosphorylation	Ca^{++}Requirement	AA Specificity
I, II,III	Secreted	No	μM	No
IV	Cytosolic	Yes	nM	Yes
V	Cytosolic	?	No	?

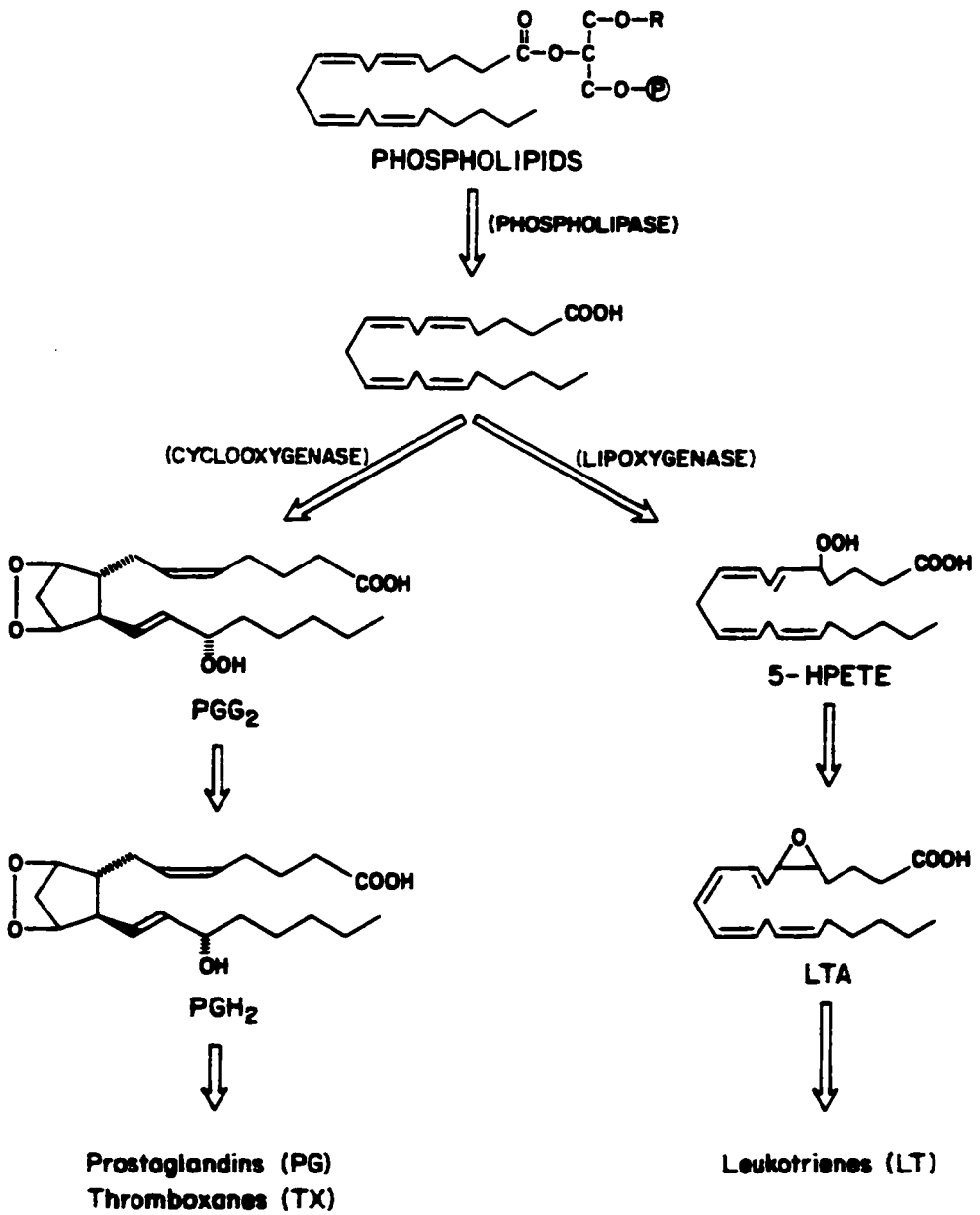
b. Cyclooxygenases

The liberation of intracellular, unesterified arachidonic acid (AA) is followed by its enzymatic oxidation in at least two separate pathways (Fig.3). One pathway, catalyzed by prostaglandin synthetase (also termed cyclooxygenase; COX), leads to the formation of prostaglandins and thromboxanes. The initial diperoxide formed in this reaction is referred to as PGG_2. This intermediate rapidly loses the peroxide at the 15-carbon position to form PGH_2 due to the inherent peroxidase activity of cyclooxygenase (Behrman & Romero, 1991). There are two isoforms of COX, COX I and COX II, which exhibit the same catalytic function but are encoded by different genes (Kujubu et al., 1991, 1993). COX II was first identified from analysis of immediate early response genes in murine and chick cells (Kujubu et al., 1993; Simmons et al., 1989) and was thus considered an inducible COX, as in contrast to the constitutive form, COX I. COX II has a larger mRNA transcript than COX I, largely due to the long 3'-untranslated region containing multiple AUUUA sequences. These sequences have been associated with rapid degradation of mRNA and are characteristic of the immediate early response genes that are induced by mitogens (Shaw and Kamen 1986).

c. Lipoxygenases

Another pathway of arachidonic acid oxidation is catalyzed by lipoxygenase. Lipoxygenase-catalyzed oxidation of arachidonic acid leads to noncyclized, hydroxylated derivatives at the 5-carbon position. The products of this pathway include leukotriene D_4 (LTD_4), 5-hydroxy-eicosatetraenoic acid (HETE), and leukotriene B_4 (LTB_4; Fig. 3). There are other hydroxylated products of arachidonic acid in addition to the compounds hydroxylated at carbon 5. These include 12-HETE as well as 11- and 15-HETEs (Behrman & Romero, 1991).

Figure 3: **Pathways of arachidonic acid metabolism.**


PHOSPHOLIPIDS
(PHOSPHOLIPASE)
COOH
(CYCLOOXYGENASE)
(LIPOXYGENASE)
COOH
OOH
PGG2
OOH
COOH
5-HPETE
COOH
OH
PGH2
O
COOH
LTA
Prostaglandins (PG)
Thromboxanes (TX)
Leukotrienes (LT)

2. Physiological functions of eicosanoids

a. Prostaglandins (PGs)

Cyclooxygenase-catalyzed oxidation of arachidonic acid results in production of prostaglandins and thromboxane (Fig. 4). Thromboxane (TX) A_2 is mainly synthesized in platelets and is a potent platelet-aggregating substance and vasoconstrictor. TXA_2 has a very short life and is degraded rapidly in the presence of water into TXB_2 (Hamberg et al., 1975). Prostacyclin or PGI_2 is produced in arterial and other tissues (Sun et al., 1977; Fenwick et al., 1977). PGI_2 is both an effective inhibitor of platelet aggregation and a vasodilator; these properties are directly antagonistic to those of TXA_2 (Fenwick et al., 1977).

PGD_2 is formed from PGH_2 by 11-keto-isomerase. This isomer of PGE_2 has been identified in brain and platelets, but the precise physiologic role of this compound has not been determined (Lands, 1979). PGE_2 and $PGF_{2\alpha}$ are produced by virtually every tissue, and substantial amounts are seen in the mammalian ovarian follicle (LeMaire et al., 1973) and uterus (Horton and Poyser, 1976). PGE_2 and $PGF_{2\alpha}$ play important roles in biological processes such as mitogenic signalling (Fagot et al., 1993; Garrone et al., 1994; Huang et al., 1991; Goin et al., 1993; Nolan et al., 1988). In the ovary, PGE_2 and $PGF_{2\alpha}$ are believed to be involved in regulating a variety of ovarian functions including steroidogenesis, ovulation and luteolysis (Behrman and Romero 1991; Gore-Langton and Armstrong, 1988; Lipner, 1988; Niswender and Nett, 1988). In the egg-laying hen, PGs are important in initiating oviposition (Hertelendy and Biellier, 1978; Rzasa, 1978; Shimada and Asai, 1979; Hammond et al., 1980) and the stimulation of uterine contraction (Wechsung and

Houvenaghel, 1980). Although the endocrinecontrol of ovarian PG secretion, particularly by gonadotropin, has been extensively examined in many species (Lipner, 1988; Tsang et al., 1988), the role of intra-ovarian growth factors such as TGFα and TGFβ in the regulation of PG secretion by granulosa cells remains to be defined.

b. Leukotrienes

LTD_4 is identical to the slowly reacting substance of anaphylaxis, SRS-A (Behrman and Romero 1991). HETE shows chemotactic activity for human neutrophils and eosinophils (Behrman & Romero,1991). The physiological function of other leukotrienes such as 12-HETE, as well as 11- and 15-HETEs is not clear. Considerable interest has been directed to a possible role of the hydroxylated products as endogenous inhibitors of thromboxane synthesis. Leukotrienes are present in the mammalian ovary, and the production of LTB_4 is stimulated by hCG (Yoshinaga et al.,1994).

c. Lysophosphatidyl choline

Phospholipase A_2 metabolizes membrane lipids to arachidonic acid (AA) and lysophosphatidyl choline (LPC), which is further converted to lysophosphatidic acid (LPA) by phospholipase D (PLD). Although both LPC and LPA have been demonstrated as potent mitogens in a variety of cell types (Tigyi et al., 1994; Durieux and Lynch 1993), their role in granulosa cells is unknown.

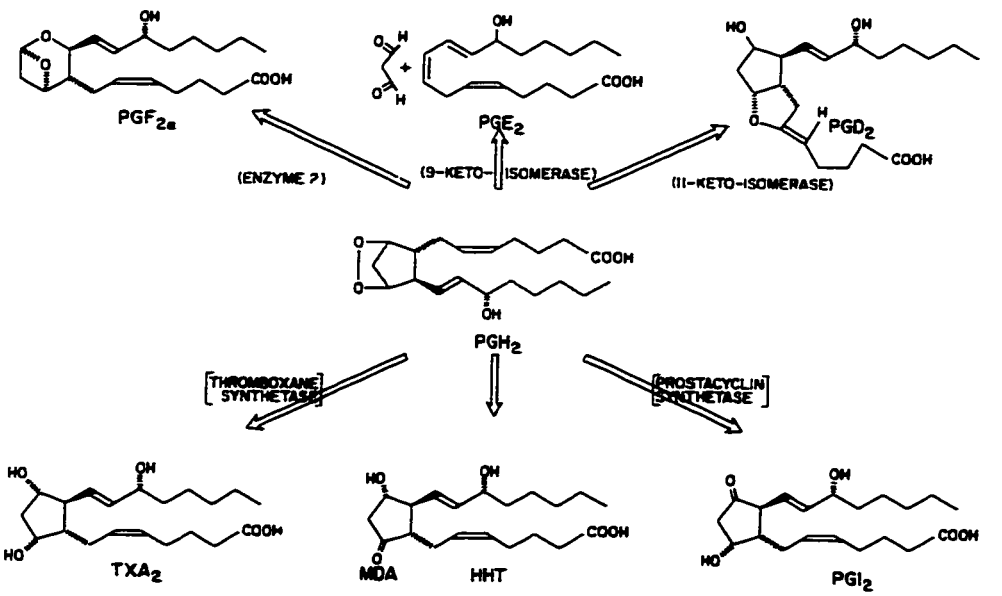
d. Prostaglandin receptors

The biological effects of PGE_2 and $PGF_{2\alpha}$ are mediated through their interaction with specific receptors. There are at least three receptor subtypes for PG of the E series (termed EP1, EP2, and EP3), which have been defined on the basis of their different pharmacological

profiles and signal transduction pathways. Activation of EP1 is coupled to stimulation of phospholipase C, and thus cytosolic Ca^{++} mobilization, while activation of EP2 and EP3 results in stimulation and inhibition of adenylate cyclase, respectively (Yumoto et al., 1986a & b). PGF receptor (FP receptor) is also believed to be coupled to phospholipase C and is involved in the regulation of intracellular Ca^{++} mobilization (Davis et al., 1987).

Peroxisome proliferator-activated receptor (PPARs) are nuclear hormone receptors that regulate gene transcription in response to peroxisome proliferators and fatty acids. PPARs play important roles in the regulation of adipocyte differentiation (Yu et al., 1995) and COX II expression in immortalized mouse liver cells (Ledwith et al., 1997). It has been reported that PGA1, PGA2, PGD1, PGD2, and PGJ2 are capable of activating all three PPAR subtypes (PPARα, PPARδ and PPARγ), however, PGE and PGF are ineffective (Yu et al., 1995). It is unknown whether PPARs are present in the ovarian cells and whether PG action is involved in these nuclear mechanisms.

Figure 4: Biosynthesis of prostaglandins and thromboxanes

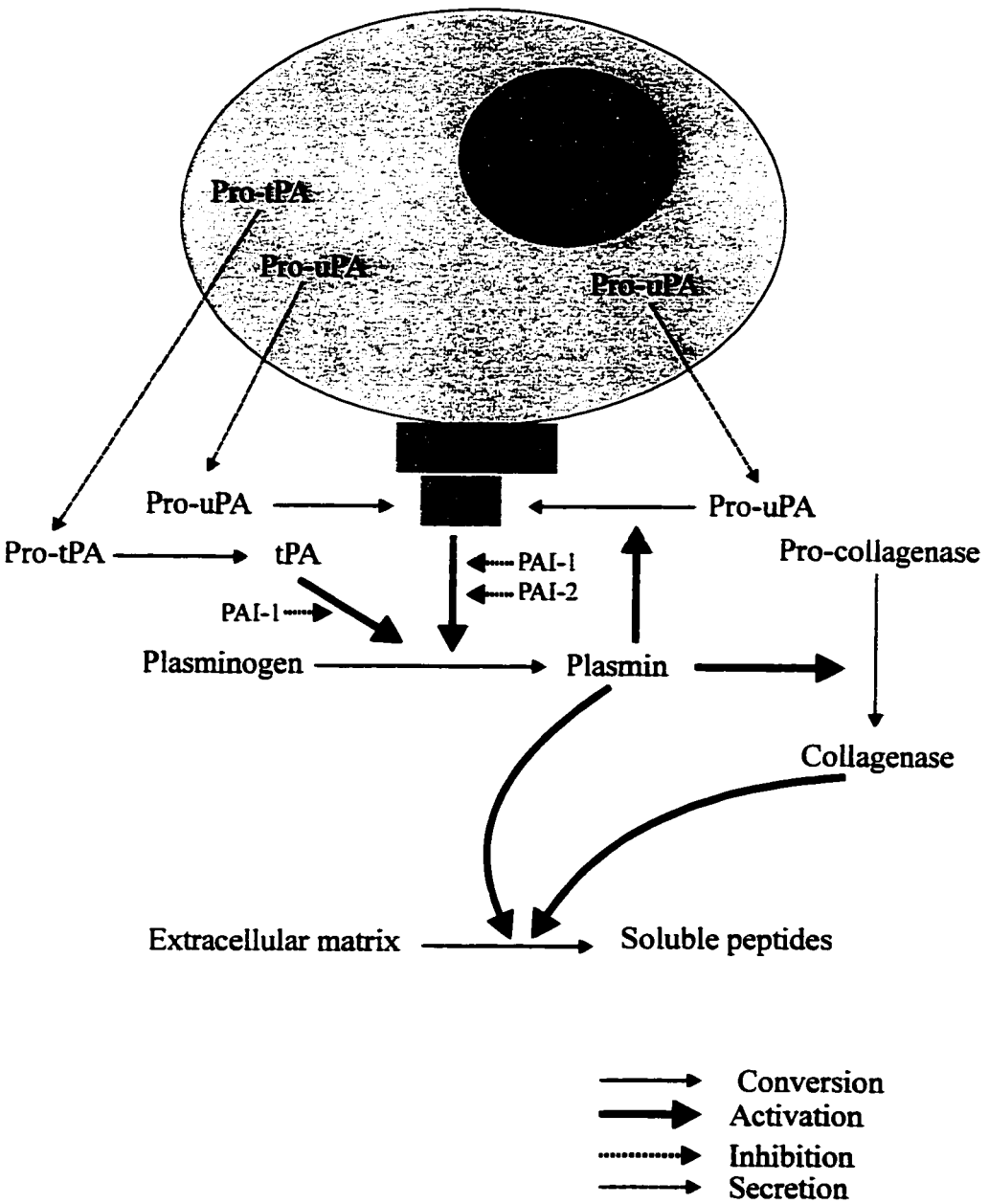

OH
COOH
PGF2a
PGE2
HO
PGD2
(ENZYME ?)
(9-KETO-ISOMERASE)
(11-KETO-ISOMERASE)
PGH2
THROMBOXANE SYNTHETASE
PROSTACYCLIN SYNTHETASE
TXA2
MDA
HHT
PGI2

G. Plasminogen activator (PA) system in the ovary

Extracellular proteolytic activity is required in many biological processes involving tissue remodelling (Strickland and Beers, 1976), tissue destruction (Ossowski et al., 1976; Busso et al., 1989) and cell migration (Strickland and Reich, 1976; Ossowski and Reich, 1983). The PA system is a general system for providing such extracellular proteolysis. Although this system has been thought primarily as one responsible for dissolving blood clots, it is now clear that the same components are involved in many other biological processes that require extracellular proteolysis including mammary gland involution (Busso et al., 1989), blastocyst implantation (Sappino et al., 1989), tumor metastasis (Ossowski and Reich, 1983) and ovulation (Beers et al., 1975; Strickland and Beers, 1976).

As shown in Fig. 5, the PA system comprises plasminogen, two types of PA (urokinase-type and tissue-type PA), various PA inhibitors (PAI-1 and PAI-2), plasmin and PA receptors (Robbins et al., 1967; Loskutoff et al., 1988; Nielsen et al., 1988). The regulation of the PA system is complex and occurs at many levels. Since plasminogen is present abundantly in plasma and body fluids (Bachmann, 1987), the regulation of this system must be mainly at the level of synthesis and activity of the rate-limiting PAs as well as the specific PAIs. The PA receptors are important for the localization of the proteolytic activity at the site(s) of tissue remodelling, which function in the overall control of the PA system. Together this multi-level and precise regulatory mechanism appears to be important in fine-tuning the expression and function of the PA system and provides the controlled proteolytic activity that characterizes many physiologic and pathologic processes.

Figure 5: The plasminogen activator system.


Pro-tPA
Pro-uPA
Pro-uPA
Pro-uPA
Pro-uPA
Pro-tPA
tPA
PAI-1
PAI-2
PAI-1
Pro-collagenase
Plasminogen
Plasmin
Collagenase
Extracellular matrix
Soluble peptides
Conversion
Activation
Inhibition
Secretion

1. Components and Physiological Functions

a. Plasminogen

Plasminogen is present in plasma and extracellular fluids at a concentration of 1 to 2 μM. It associates with fibrin and other proteins via lysine-binding sites located in the kringles of its noncatalytic portion (Plow and Miles, 1990) and is activated by PA to form plasmin. Whereas the "classical" plasmin substrate is fibrin, it is now apparent that other matrix proteins can also be cleaved by this protease. In addition, plasmin is one of the activators of collagenase and metalloproteinase precursors, and thus plays a pivotal role in extracellular matrix remodelling (He et al., 1989).

b. Plasminogen activators

Plasminogen activators are serine proteases that convert the zymogen plasminogen to the active trypsin-like protease plasmin. Two types of PA [tissue-type (tPA) and urokinase (uPA)] have been identified. They are the products of distinct genes which differ mostly in the domain organization and function of their noncatalytic regions. Both PAs are secreted as single-chain proteins (Dano et al., 1985). The two PAs have distinct targeting determinants in their noncatalytic regions: the "growth factor domain" of uPA directs the binding of the enzyme (and that of pro-uPA) to a plasma membrane receptor (Vassalli et al., 1985; Blasi et al., 1990) while the structural domains in tPA (the "finger" region and the "kringles") allow tPA binding to fibrin and other components of the extracellular matrix (ECM; Gething et al., 1988). These interactions could have a dramatic effect on the focusing of PA-controlled proteolysis (Andrade-Gordon and Strickland, 1986).

Tissue-type PA has been purified from various sources, including heart, kidney,

ovary, uterus, plasma and conditioned cell culture medium (Granelli-Piperno and Reich, 1978; Rijken et al., 1981; Levin and Loskutoff, 1982). The genes encoding the human, porcine, mouse, rat and chicken tPA have been identified (Ny et al. 1984; Fisher et al., 1985; Degen et al., 1986; Heaton and Gelehrter, 1990; Rickles et al., 1988; Johnson et al., 1997).

Although uPA was first discovered in human urine, the presence of this PA type in other body fluids and tissues is now well recognized. It is present in blood plasma, seminal plasma, the prostate gland and the ovary (Ptosed et al., 1982; Propping et al, 1978; Ny et al., 1985). Both the high and low molecular weight active forms of uPA have been identified in various vertebrate cell systems. These isoforms share an identical B-chain which contains the active sites and differs in the molecular weight due to limited proteolytic cleavage of the 24 kDa A chain of the high molecular weight urokinase, leaving a 21 amino acid-polypeptide attached to the ~30 kDa B-chain (Saksela 1985).

The gene coding for uPA has been identified in tissues of many species (Verde et al., 1984; Nagamine et al., 1984; Belin et al., 1985; Zhang et al., 1994; Leslie et al., 1990). The cDNA sequence of uPA from these species shows extensive homology. The human 6.4-kb uPA gene contains 11 exons (Riccio et al., 1985) and codes for the protein via 2.5-kb mRNA (Verde et al., 1984). The functional and structural domains for uPA are one kringle structure and the growth factor-like domain (Riccio et al., 1985). It lacks the finger domain present in tPA and the second kringle structure which gives the characteristic fibrin-binding property of tPA, and thus the affinity to fibrin. The growth factor-like domains of uPA and tPA are structurally similar to the receptor-binding regions of EGF and TGFα (Gunzler et al., 1982; Derynck et al., 1984; Komoriya et al., 1984). Whereas the function of this domain

is unknown in tPA, the growth factor domain of uPA contains the receptor-binding amino acid sequence (Appella et al., 1987). Plasmin, which permits uPA binding to its receptors, further processes uPA to a 33-KDa molecule that lacks the growth factor domain (receptor-binding) and kringle domain, but is still fully active (Stump et al., 1986).

c. Urokinase PA receptors

One of the interesting aspects of the PA system is the putative role of the high-affinity membrane receptor for uPA (uPAR; Vassalli et al., 1985, Stoppelli et al., 1985). The receptor has been purified and appears to be a 55-60 KDa glycosylated protein (Nielsen et al., 1988). The binding region in uPA has been localized to the cysteine- rich part of the growth factor domain of uPA (Appella et al., 1987). Despite the partial amino acid homology between this sequence and EGF, the binding of uPA to the receptor is not inhibited by EGF. A synthetic peptide with the same amino acid sequence as the growth factor domain of uPA (residues 12-32) competes with uPA for binding to uPAR (Appella et al., 1987). This sequence differs from the respective sequence in tPA, which may account for the lack of tPA binding to the receptor. The growth factor domain-defective 33 KDa uPA generated by digestion with plasmin does not bind to the receptor.

Receptor-bound uPA stimulates limited cell growth (Kirchheimer et al., 1987). This effect was demonstrated in a carcinoma cell line expressing the receptor but that did not synthesize uPA. Incubation of cells with uPA induced cell proliferation, whereas inhibition of the enzymatic activity with diisopropylfluorophosphate (which does not interfere with the receptor binding) abolished the mitogenic response. These findings suggest that the uPA-uPAR interaction may stimulate cell division through an intracellular signalling

mechanism.

Receptor-bound uPA may have an important role in cell invasion and metastasis, processes often correlated with increased uPA activity. Since uPA is first secreted into the extracellular space and is then bound to uPAR, cells not secreting uPA but possessing the receptor may bind enzyme produced by other cells and thereby utilize the uPA-mediated proteolytic machinery (Huarte et al., 1987). The presence and physiological role of uPA receptor(s) in chicken is not known.

d. PA Inhibitors

Plasminogen activator inhibitor-1 (PAI-1) is a single-chain polypeptide of approximately 54 KDa that was initially characterized as an endothelial cell PA inhibitor (Van Mourik et al., 1984). Kinetic studies have shown that PAI-1 is the most efficient inhibitor known for both uPA and tPA (Loskutoff et al., 1988).

The gene coding PAI-2 has been isolated (Ye et al., 1987). PAI-2 was originally described as a uPA inactivator in the placenta (Kawano et al., 1970; Astedt et al., 1985). The secretion of PAI-2 varies between different cell types and is modulated by various growth promoting factors (Genton et al., 1987; Wohlwend et al., 1987).

2. Regulation of PA in the ovary

The molecular and cellular mechanisms involved in the development of ovarian follicles are coupled to tissue remodelling processes needed for the successful growth and eventual ovulation. It seems that the proteolytic activity needed for these processes may involve regulation of PA (Liu et al., 1991). It has been shown that the level of tPA mRNA is low in granulosa and theca cells but high in the oocyte in early stages of follicular development (Cajander et al., 1989; Peng et al., 1993). However, preovulatory follicles

demonstrate high levels of tPA activity with decreased level of PAI (Peng et al., 1993). Various intraovarian factors such as EGF, TGFα, IGF and relaxin have been shown to modulate rat granulosa cell PA activity (Too et al., 1984; Galway et al., 1989).

While two types of PA have been identified in granulosa and theca cells in the domestic hen (Tilly and Johnson 1987; Politis et al., 1990), the predominant PA activity in granulosa cells is uPA (Lafrance et al., 1993a). The activity of the enzyme in avian granulosa cells from the largest preovulatory follicle (F1) is known to be suppressed by LH and to decrease before ovulation (Tilly and Johnson, 1987; Tilly et al., 1990b; Jackson et al., 1993), indicating that the enzyme may be less important for follicle rupture than has been reported in mammals (Beers et al., 1975; Riech and Miskin,1985; Liu et al., 1987; Tsafriri et al., 1989). However, observations on the dynamic changes on avian PA activity have suggested that the enzyme may be involved in the ovarian tissue remodelling accompanying cell proliferation during follicular growth (Politis et al., 1990; Tilly et al., 1992b). Although several studies have demonstrated that TGFα stimulates PA activity in hen granulosa cells (Lafrance et al., 1993a&b; Tilly and Johnson , 1990b) and that the extent of this stimulation is dependent on follicular development (Lafrance et al., 1993a&b), little is known regarding the mechanism of this regulation.

II. RATIONALE AND DEFINITION OF RESEARCH PROBLEMS

Ovarian follicular development involves granulosa cell and theca cell proliferation, differentiation and follicular tissue remodelling (Lafrance et al., 1993a & b), and is regulated by complex actions and interactions of gonadotropins and various intraovarian factors (steroids, growth factors and cytokines), among which, those of gonadotropins and steroids have been well documented. Although much is known concerning the production and action of growth factors in the ovary, the present knowledge regarding their mechanisms of action remains incomplete. The mitogenic signal of TGFα and EGF is transduced upon receptor binding which then triggers a series of intracellular changes. These include alterations in tyrosine kinase activity (Moran et al., 1990), ionic fluxes (Moolenaar et al., 1986) and arachidonic acid metabolism (Harris et al., 1990; Unemori et al., 1994; Refsnes et al., 1994; Glasgow et al., 1992; Handler et al., 1990). Precisely how, if at all, these EGF-regulated biochemical processes elicit the mitogenic responses remains to be determined.

Prostaglandins (PG) are believed to play important regulatory roles in a variety of ovarian functions including steroidogenesis, ovulation and luteolysis (Behrman and Romero, 1991; Gore-Langton and Armstrong, 1988; Lipner, 1988; Niswender and Nett, 1988). In the egg-laying hen, PGs are involved in initiating oviposition (Hertelendy and Biellier, 1978; Rzasa, 1978; Shimada and Asai, 1979; Hammond et al., 1980) and stimulating uterine contractions (Wechsung and Houvenaghel, 1980). The role of intra-ovarian growth factors such as TGFα and TGFβ in the regulation of PG secretion by granulosa cells remains to be defined.

Although considerable attention has been focused on the role of PG in the control of follicular rupture in the mammalian ovary (Ainsworth et al., 1979; Tsang et al., 1979; Tsafriri et al., 1973; Murdock et al., 1986), whether their secretion and action are related to the activation of the follicular wall remodelling process is equivocal (Strickland and Beers, 1976; Liu and Stephen, 1988; Shimada et al., 1983; Espey et al., 1985). In the domestic hen, plasminogen activator (PA) activity in cultured granulosa cells from the largest preovulatory follicle is stimulated by PGE_2 (Tilly and Johnson, 1987), suggesting that PG may be involved in the regulation of PA activity in the follicle destined to ovulate. Previous studies from our laboratory have shown that TGFα and TGFβ play important roles in the regulation of granulosa cell proliferation and PA activity during follicular development (Lafrance et al., 1993a & b). Whether these changes are mediated by PG secretion and action needs to be clarified.

Several investigations have suggested that by-products of arachidonic acid synthesis and arachidonate metabolites are key elements in the regulation of cell proliferation (Handler et al., 1990; Fagot et al., 1993; Garrone et al., 1994; Huang et al., 1991; Goin et al., 1993). Mitogenic signalling by EGF in BALB/c3T3 cells requires the metabolism of arachidonic acid to prostaglandins (Handler et al., 1990) while, in the regulation of Syrian hamster embryo fibroblast proliferation, linoleate metabolites of the lipoxygenase pathway, but not prostaglandins, are important (Glasgow et al., 1992). PGE_2 is also known to be antiproliferative in guinea pig tracheal smooth muscle cells (Florio et al., 1994). In addition, both LPC and LPA have been demonstrated as potent mitogens in a variety of cell types (Tigyi et al., 1994; Durieux and Lynch, 1993).

Whether these arachidonic acid by-products and metabolites play a role in the mitogenic response of ovarian cell to EGF or TGFα is unknown.

One of the key enzymes involved in the synthesis of PG is prostaglandin endoperoxide synthase, also known as COX. Two isoforms of COX have been identified; an inducible form COX II and a constitutive form, COX I. Although COX II is induced in preovulatory rat and bovine follicles in response to the ovulatory LH surge, this induction is specifically localized in the granulosa cells and believed to be involved in the control of follicular rupture (Hedin et al., 1987; Wong et al., 1989; Wong and Richards, 1991; Sirois, 1994; Sirois and Richards, 1992). Whether COX II is involved in the regulation of granulosa cell PG production by growth factors during follicular development remains to be investigated.

Cytosolic PLA_2 is a key enzyme involved in the release of arachidonic acid and subsequent production of prostaglandins (PGs), which play an important role in regulating ovarian function, including mitogenic signalling by TGFα. The binding of EGF to its receptor triggers a series of intracellular changes including alterations in tyrosine kinase activity believed to be essential for mitogenic signalling (Moran et al., 1990). In addition, EGF/TGFα receptor signalling also includes a rise in intracellular Ca^{++} concentration and MAP kinase activation (Tahar et al., 1995; Rosen and Greenberg, 1996), which are essential for the activation of $cPLA_2$. It will be important to study whether $cPLA_2$ is involved in the regulation of PG production by TGFα and TGFβ during follicular maturation.

TGFβ, a secretory product of hen granulosa and theca cells throughout follicular

development (Law et al., 1995), appears to act antagonistically with TGFα in the regulation of granulosa cell functions (Li et al., 1994; Skinner et al., 1987; Kim and Schomberg, 1989; Dobson and Schomberg, 1987). The binding of TGFβ to its receptor results in the formation of a heteromeric receptor complex which is activated via phosphorylation (Massague et al., 1996b; Wrana et al., 1994). One of its signalling proteins, MADR2, is then phosphorylated by the type I receptor and is translocated to the nucleus, presumably to act as transcriptional regulator (Attisano and Wrana, 1996). Previous work from our laboratory has demonstrated that TGFβ regulation of PA activity in hen granulosa cells is dependent on follicular maturation (Lafrance et al., 1993a). The reason(s) for this developmental dependency of TGFβ action is not clear, nor is it known if the follicular stage-specific regulation of MADR2 expression by this growth factor may be a determinant of the relative responsiveness of this signalling pathway to TGFβ. To date, the presence and regulation of MADR2 in the ovary by TGFβ have not been examined.

Plasminogen activators (PAs) are serine proteases that convert plasminogen to plasmin. Plasmin-mediated proteolytic mechanisms are involved in numerous biological processes requiring extracellular matrix degradation during tissue growth and remodelling (Dano et al., 1985a). While both tPA and uPA have been identified in granulosa and theca cells of the domestic hen (Tilly and Johnson, 1987; Politis et al., 1990; Armstrong et al., 1990), the predominant PA activity in granulosa cells is uPA (Lafrance et al., 1993a). Observations on the dynamic changes on avian PA activity have suggested that the enzyme may be involved in the ovarian tissue remodelling accompanying cell proliferation during follicular growth (Politis et al; 1987; Tilly et al., 1992b). Although several studies have

demonstrated that TGFα stimulates PA activity in hen granulosa cells (Lafrance et al., 1993a & b; Tilly and Johnson, 1990b) and that the extent of this stimulation is dependent on follicular development (Lafrance et al., 1993a & b), little is known regarding the mechanism of this regulation.

III. OVERALL OBJECTIVES

The overall objective of my thesis research is to study the mechanism of action of eicosanoids in the regulation of mitogenesis and uPA activity in granulosa cells by TGFα during follicular development.

IV. HYPOTHESIS

The increase in granulosa cell PG production in response to TGFα plays an important role in the mitogenic action of TGFα and also mediates TGFα action in the regulation of PA activity. The increase of PG production is due to follicular stage-dependent regulation of both phosphalipase A_2 and cyclooxygenase at the transcriptional and translational levels. The regulation of granulosa cell PG production is modulated by TGFβ, possibly via up-regulation of its signaling protein, MADR2.

V. SPECIFIC AIMS

A. To study the regulation PG production by TGFα and TGFβ during follicular development and if this regulation is dependent on PG production.

B. To determine whether TGFα-induced granulosa cell DNA synthesis is mediated by PGs during follicular development.

C. To investigate whether COX II is important in the signalling cascade for TGFα in the regulation of granulosa cell PG production, and thus DNA synthesis, during follicular development.

D. To determine whether the regulation of PG production by TGFα and TGFβ is also at the level of $cPLA_2$.

E. To study whether the autoregulation of MADR2 by TGFβ may be important in

conferring follicular-stage dependent suppression of granulosa cell $cPLA_2$ by the growth factor .

F. To determine whether urokinase plasminogen activator (uPA) is up-regulated by TGFα.

VI. MATERIALS AND METHODS

A. Reagents

Media and culture reagents were purchased from Gibco/Bethesda Research Laboratories (Burlington, Ontario, Canada). Collagenase-1A, trypsin (type I), thrombin (from bovine plasma), DNA (calf thymus, type XV), Hoescht 33258, fibrinogen (type IV), agarose (low gelling temperature, type VII), and Triton X-100 were purchased from Sigma Chemical Co. (St. Louis, MO). Recombinant human TGFα and human TGFß1 were purchased from Collaborative Research (Bedford, MA). Acrylamide (electrophoresis grade), N,N-methylene-bis-acrylamide, ammonium persulfate, tetramethylethylene diamine, dithiothreitol, and glycine were obtained from Bio-Rad (Richmond, CA). HEPES, sodium dodecyl sulfate, and bromophenol blue were purchased from BDH (Toronto, Ontario, Canada). 2-CP-amylcinnamoyl, amino-4-chlorobenzoic acid, aristolochic acid, dimethyleicosadienoic acid, nordihydroguaiaretic acid (NDGA), indomethacin, prostaglandins (PGE_1, PGE_2, $PGF_{1\alpha}$ and $PGF_{2\alpha}$), leukotrienes (LTB_4, LTC_4, LTD_4) and 5-HETE were obtained from Sigma Chemical Co.(St. Louis, MO). Naproxen and Ibuprofen were purchased from BIOMOL Research Laboratories, Inc (Plymouth Meeting, PA). [Methy-^{3}H]thymidine was purchased from Amersham Life Science (Oakville, Ontario, Canada) while [5, 6, 8, 9, 11, 12, 14, 15-^{3}H(N)]-$PGF_{2\alpha}$ and [3, 6, 8, 11, 12, 14, 15-^{3}H(N)]-PGE_2 (100-200 Ci/mmol) were purchased from Dupont/NEN (Boston, USA). 3-[1-(4-chlorobenzyl)-3-t-isopropyl-indol-2-yl]-2-2-dimethylpropanoic acid (MK-886) was a gift from Dr. A.W. Ford-Hutchinson (MERCK FROSST Canada Inc, Pointe-Claire, Quebec, Canada). RNeasy Total RNA Kit was purchased from

Qiagen (Germany). Random Primed DNA Labeling Kit was from Boehringer Mannheim (Germany), α-^{32}P dCTP and ECL Western blotting detection kit were purchased from Amersham (Oakville, Canada). Zeta-probe blotting membrane, trans-blot supported nitrocellulose membrane and Bio-Rad protein assay kit were from Bio-Rad Laboratories (Hercule,CA). X-ray films were from Eastman Kodak company (New York). The 2.8 Kb hen COX II cDNA probe was purchased from Oxford Biochem (Michigan, USA).

Hen COX I cDNA probe and polyclonal hen COX II antibody were produced in DL Simmons' laboratory (Brigham Young University, Utah, USA). The 2.8 kb hen $cPLA_2$ cDNA probe and polyclonal human $cPLA_2$ antibody were gifts from Dr. L-L. Lin (Genetics Institute, Cambridge, MA). Human MADR1, MADR2 cDNA probes and polyclonal antibodies were prodvided by Dr. J.L. Wrana (University of Toronto, Toronto, Canada). Human polyclonal EP3 receptor antibody was a gift form Dr.S.K. Dey (University of Kansas, USA). PGE_2 and $PGF_{2\alpha}$ antisera were gifts from Dr. N.R. Mason (Lilly Research Laboratories, Indianapolis, IN) and Dr. D.T. Armstrong (University of Western Ontario, London, Ontario, Canada), respectively. The chicken uPA cDNA probe was provided by Dr. J.L. Degen (University of Cincinnati, Ohio), and polyclonal rabbit anti-hen uPA antibody was a gift from Dr. J.P. Quigley (State University of New York, Stony Brook, NY).

B. Methods

1. Isolation and culture of granulosa cells

White Leghorn hens (Agriculture Canada, Nepean, Ontario, Canada) in their first year of lay were caged individually in a windowless, air-conditioned room with a 14h-light, 10h-dark cycle. The birds had free access to feed and water. The time of ovulation was predicted from the time of the previous oviposition on the basis that the former occurs 15-75 min from the latter. Approximately 10-14 h before the expected time of the next ovulation, hens were killed by cervical dislocation. The ovaries were excised and placed in ice-cold Medium 199 supplemented with HEPES (25mM), pH 7.4. Follicles from three to four hens were grouped together according to the stage of development as follows: the largest preovulatory (F1), the third largest (F3), and the fifth and sixth largest (F5-6) follicles. Granulosa cells from each group of follicles were isolated, as described previously (Asem et al., 1984), and dissociated by incubation for 15 min at 37°C in 2 ml Medium 199-HEPES containing 540 U collagenase and 0.2 mg trypsin inhibitor/follicle. Dispersed granulosa cells ($3x10^5$) were incubated in 16mm tissue culture wells (Falcon, Becton Dickinson Labware, Lincoln Park, NJ) in 0.5 ml Minimum Essential Medium supplemented with L-glutamine (0.29 mg/ml), nonessential amino acids (0.1mM), penicillin (100 U/ml), and streptomycin (100 µg/ml) at 39°C under an atmosphere of 5% CO_2 and 95% air. After an incubation period of 3 h to allow cell plating, the medium was changed and the test agents were added, then the cells were cultured for an additional 21 h. At the end of the culture period, the medium was collected, and 200 µl aliquots were diluted with 1 ml ethanol for PG assay. Triton X-100 was added to a final concentration of 0.025% to the

remaining medium (300µl) for PA assays. The aliquots of medium were stored at -20°C pending assays for PG and PA activity. Fresh Minimum Essential Medium containing 0.025% Triton X-100 (0.5 ml) was added to each culture well, and the cells were removed from the wells with a rubber policeman. The cell suspensions were sonicated in polypropylene microfuge tubes for 5 sec, using a Fisher Sonic Dismembrator (model 300, Fisher Scientific, Fair Lawn, NJ) set at a relative output of 35. The cell samples were aliquoted and store at -20°C until assayed for PA activity. A 100-µl aliquot of each cell sample was diluted with an equal volume of 0.05 M phosphate buffer (pH 7.4) containing NaCl (2M) and ethylenediaminetetraacetic acid (EDTA,2mM) for DNA determination. Cell viability, determined by the trypan blue dye exclusion test (Simmons et al., 1976) at each stage of follicular development, was greater than 90% before and after culture.

2. *Determination of net PA activity*

The net PA activities of the cell (PAc) and medium (PAs) fractions were determined by a fibrinolysis assay adapted from a method described previously (Ailenberg et al., 1990). Briefly, 96-well microtiter plates precoated with an iodinated fibrinogen solution ($1x10^5$ cpm; 2µg/well) were treated with thrombin (2mM) for 1 h at 37°C for the conversion of fibrinogen to fibrin. The wells were then washed three times each with 250µl Tris-HCl buffer (0.1 M; pH8.1). The assay was carried out in a final volume of 250 µl Tris-HCl buffer containing 0.2 µg chicken plasminogen per well [purified from chicken plasma by the lysin-Sepharose 4B affinity chromatography method (Deutch and Mertz, 1970)], and the plates were incubated for 4 h at 39°C. Samples were also tested without plasminogen to evaluate the plasminogen dependence of their fibrinolytic activity. Basal and maximum

releases of iodinated fibrinogen were determined using plasminogen alone and in the presence of 2.5% (wt/vol) trypsin, respectively. At the end of the 4h incubation, aliquots of the supernatant were counted to determine the radioactivity released. The intra- and inter-assay coefficients of variation were 2.6% and 9.8%, respectively. PA activity was expressed as cpm/μg DNA.

3. Zymographic analysis of uPA activity

PA activity was characterized by zymography as described previously (Granelli-Piperno & Reich, 1978). Briefly, protein samples from both cell and medium were separated on 9% non-reducing one-dimensional sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) at 7.5 mA per gel until the bromophenol blue tracking dye migrated out of the gel. The gel was then washed twice with 2.5% (w/v) Triton X-100, overlaid onto a 1% low melting point agarose indicator gel containing chicken plasminogen (30 μg/ml), fibrinogen (2mg/ml) and thrombin (0.2 units/ml), and incubated for 24-36 h at 37°C in a humidified incubator to allow PA to diffuse into the indicator gel and lyse the fibrin.

4. DNA determination

Cellular DNA content was determined by a fluorometric method, using the Hoescht 33258 indicator dye (Cesarone et al., 1979; Labarca and Paigen, 1980) and calf thymus DNA as standard. One milliliter of Hoescht 33258 (0.1 μg/ml) was added to each cell sample, and the mixture was incubated for 5 min in the dark. The fluorescence was monitored in a Perkin-Elmer LS-5 Luminescence Spectrometer (Norwalk, CT), with excitation and emission wavelengths set at 356 and 457 nm, respectively. The sensitivity of the assay was 10 ng/ml, and the assay was linear up to 5 μg/ml.

5. PG Determinations

Duplicate sample aliquots of appropriate size were analyzed for PGE and PGF by RIA according to the method of Jaffe et al. (1973), using PGE_2 and $PGF_{2\alpha}$ as standards. Radiolabelled PG and corresponding antibodies were dissolved in phosphate-buffered saline-gelatine solution [PBSG: sodium phosphate dibasic (76 mM), sodium phosphate monobasic (20 mM), sodium chloride (154 mM), EDTA (1.27 mM), sodium azide (2.92 mM) and gelatin (1 g/L); pH 6.9]. Appropriate volumes of sample which were in 90% ethanol were evaporated and dissolved in PBSG. The total assay volume was 300 µl: 100 µl of PG standards (0, 5, 10, 20, 40, 80, and 160) or sample, 100 µl of radiolabelled PG and 100 µl of antibody (at a concentration to achieve 20-40% binding of the tritiated ligand). The assay mixture was vortexed gently and incubated overnight at 4°C. The tubes in the standard curve included those for the "total counts" (total counts of tritiated PG added to each assay tube), "B_0" (binding of tritiated PG to the antibody in the absence of unlabelled PG) and an assay blank which was subtracted from all values in the assay. PG standards were measured in triplicate.

Following an overnight incubation, the assay tubes were placed on an ice bath and 750 µl of charcoal mixture [charcoal (3.57 g/L) and dextran (0.357 g/L) in PBS] were added to each tube to adsorb unbound PG. They were left on ice for 15 minutes and then centrifuged (550 x g for 15 minutes) to separate bound from free PG. The supernatant containing the antibody-PG complex was decanted into scintillation vials containing 4 ml of scintillation fluid and the radioactivity was determined in a liquid scintillation counter.

The sensitivity of both assays was 5 pg/tube. Results are expressed in ng PG per µg

DNA to allow for the comparison of treatment effect on the PG-producing activity. The cross-reactivities of the antisera have been reported elsewhere (Lewis et al., 1978; Evans et al., 1981). Since the antiserum for PGE_2 and $PGF_{2\alpha}$ cross-reacted significantly with PGE_1 (16.8%) and $PGF_1\alpha$ (125%), respectively, the values obtained are reported as PGE and PGF equivalents.

6. Solubilized cell extracts and immunoblot analysis

To prepare solubilized cell extracts for analysis of COXII and uPA, granulosa cells were sonicated (8 sec/cycle, 3 cycles) on ice in TED sonication buffer (20 mM Tris, 50 mM EDTA, 0.1 mM diethyldothiocarbamic acid, pH 8.0) containing 1% triton-100. The sonicates were centrifuged at 13,000 x g for 30 min at 4°C. The supernatant (solubilized cell extract) was stored at -20°C until electrophoretic analyses were performed. Protein concentration was determined using the Bio-Rad Protein Assay method.

Since $cPLA_2$ and MADs translocate from the cytosol to the nucleus upon activation, whole cell lysates rather than cytosolic fractions from granulosa cells were used for Western blot analysis. Total cell protein extracts were prepared as follows: granulosa cells were sonicated (8 sec/cycle, 3 cycles) on ice in 10mM HEPES buffer (pH7.4) containing 1mM EGTA and 2mM PMSF. The sonicates were stored at -20°C until electrophoretic analyses were performed. The level of protein concentration was also determined using the Bio-Rad Protein Assay method.

Equal amounts of protein (60-100 μg/lane, depending on individual experiments) present in cell extracts were resolved by one-dimensional SDS-PAGE (9%) electrophoresis, and electrophoretically transferred to nitrocellulose membrane.

Membranes were blocked with 5% non-fat milk, and subsequently incubated with polyclonal antibodies (COX II, EP3, $cPLA_2$, MADR1, MADR2 or uPA) diluted in TBS (10 mM Tris-buffered saline, pH 7.5) containing 5% milk. An ECL kit was used to visualize immunopositive protein.

7. RNA isolation and Northern analysis

Total RNA from granulosa cells was isolated by using RNeasy Kit and quantified spectrophotometrically at 260 nm. RNA samples (6-12 µg) were denatured at 55°C for 15 min in 45% formamide and 5.4% formaldehyde, and electrophoresed at room temperature in 1% formaldehyde-agarose gel. Gels were stained with ethidium bromide to confirm even loading of RNA samples (comparative staining of 28S and 18S ribosomal bands). Following transfer to a membrane, blots were UV cross-linked and hybridized to a ^{32}P-labelled cDNA probe for COX I, COX II, $cPLA_2$, MADR1, MADR2 or uPA. Each probe was labelled using a Random Primed [α-^{32} p] DNA Labelling Kit. Blots were washed twice (5 min / wash) at room temperature, 4 times (15 min / wash) at 65 °C in 0.3 x saline sodium citrate (SSC), 0.5% sodium dodecyl sulfate (SDS) and subsequently exposed to X-ray film at -80°C. Densitometric analysis of COX II and 28S rRNA bands were performed using a Molecular Dynamics Phosphoimager. The data was normalized by the respective 28S rRNA and expressed as a percentage of the control (defined as 100%). The same blot was reprobed with a differently labelled cDNA after they were stripped of radioactivity with 50% formamide (in 10 mM sodium phosphate, pH 6.5) for 1 h at 80 °C.

8. [^{3}H]Thymidine incorporation assay

[^{3}H]thymidine incorporation was assessed as described previously (Miro et al., 1991)

with minor modifications. Following the initial cell plating period, granulosa cells were cultured for an additional 18 h in the absence and presence of various test agents and [^{3}H]thymidine (0.125 μCi) was added to each well 3 h after the commencement of the treatment period. The medium was removed and the cells were washed twice with Dulbecco's PBS containing unlabelled thymidine (100 μg/ml), then subsequently washed once with Dulbecco's PBS alone. The cells were fixed for 20 min in 1 ml trichloroacetic acid (0.5%, w/v; 4°C) and washed first with trichloroacetic acid (1 ml) and then with methanol (1ml). The DNA pellets, dissolved in 0.25 ml KOH (0.5M), were adjusted to neutral pH with HCl (0.5M). A 100 μl aliquot was stored at -20°C for subsequent DNA assay and radioactivity in the remaining solution was determined.

9. Statistical analysis

Follicles from three or four birds were used for each experiment. Results were expressed as the mean ± SEM of three to six experiments each with three replicate culture wells per treatment group. Where required, data were transformed logarithmically before statistical analysis to remove heterogeneity of variance, as determined by the Barlett's test. Statistical analysis was carried out by Analysis of Variance (one-, two- and three-way). Significant differences between treatment groups were determined by the Scheffé test. Statistical significance was inferred at $P<0.05$.

VII. RESULTS

A. Regulation of PG production by TGFα and TGFβ during follicular development

1. PGE and PGF production by F1 granulosa cells is stimulated by TGFα and suppressed by TGFβ in a concentration-dependent manner

Figure 6 illustrates the influence of various concentrations of TGFα (0-10 ng/ml) and TGFβ (0-20 ng/ml) on PG secretion and PA activity in granulosa cells from the F1 follicle after 21 h of culture. TGFα stimulated PGF and PGE secretion and PA activity in a concentration-dependent manner. PA activity was significantly increased by TGFα at 0.1 ng/ml ($P<0.05$) but the stimulatory effect on PGF and PGE secretion was only observed at higher concentrations (1-10ng/ml). Maximal stimulation of PGF (2.5-fold) and PGE (1.5-fold) secretion and PA activity (15.5-fold) by TGFα was noted at 1, ≥10 and 10 ng/ml, respectively. In contrast, TGFβ, at the concentration of 4 ng/ml suppressed PGF and PGE secretion to 20% and 35% of controls, respectively, and with a significant and almost complete inhibition observed at a concentration of 20 ng/ml ($P<0.01$). Moreover, TGFβ had no effect on basal PA activity ($P>0.05$).

2. The regulation of granulosa cell PG production and PA activity by TGFα and TGFβ are dependent on follicular maturation

To investigate whether the regulation of PG secretion by the growth factors is dependent on the stage of follicular development and whether PG secretion mediates the regulation of PA activity, granulosa cells from F1, F3, and F5-6 follicles were cultured for 21 h in the absence and presence of TGFα and/or TGFβ, and PG secretion and PA activity were measured. In the absence of exogenous growth factors, the ability of granulosa cells to secrete PGF and PGE *in vitro* increased significantly with follicular maturation from the

F5-6 to F3. No further increase in PG levels was apparent as follicle development reached the F1 stage (Fig. 7). Basal PGF secretion by cells from F1, F3 and F5-6 was 2.2±0.3, 2.2±0.5 and 1.1±0.3 ng/μg DNA, respectively, and was markedly higher than the secretion of PGE. Although TGFα (10 ng/ml) significantly increased PG secretion at all stages of follicular maturation ($P<0.01$; except PGE in F5-6), the amounts of PGF and PGE secreted by the granulosa cells in the presence of the growth factor were significantly greater in the more mature follicles ($P<0.05$). TGFβ (20 ng/ml) significantly inhibited basal and TGFα-induced PG secretion; the effects on the latter (particularly on PGF secretion) were more pronounced in F5-6 cells as TGFα-induced PGF secretion was suppressed to levels significantly lower than basal levels in F5-6 (Fig. 7). In contrast, basal and TGFα-induced PA activity in granulosa cells decreased with follicular development. The observed changes in PA activity with follicular maturation in response to TGFα were predominantly associated with a protease activity within a molecular weight of 30-35 KDa, which corresponds to that of urokinase PA (Fig. 44; Jaffe et al.,1973). Both basal and TGFα-induced PA activity were markedly higher in cell (PAc) than in medium (PAs) except in the TGFα group of F5-6 (Table 3). TGFβ, however, had no significant effect on basal PA activity, irrespective of the stages of follicular maturation ($P>0.05$). Moreover, ANOVA indicated a statistically significant interaction between TGFα and TGFβ and follicular stage ($P<0.001$), brought about by a significant TGFβ-induced enhancement of TGFα-stimulated PA activity in granulosa cell cultures of F5-6 and F3 but not of F1 (Fig. 7).

Figure 6: Concentration-response studies of the effects of TGFα (α) and TGFß (β) on F1 granulosa cell PG secretion and PA activity in 21 h-incubation. PA activity is the sum of PA activity in the cell (PAc) and medium (PAs) fractions. Values represent the means±SEM of four replicate experiments. * $P<0.05$, ** $P<0.01$ (compared to control)

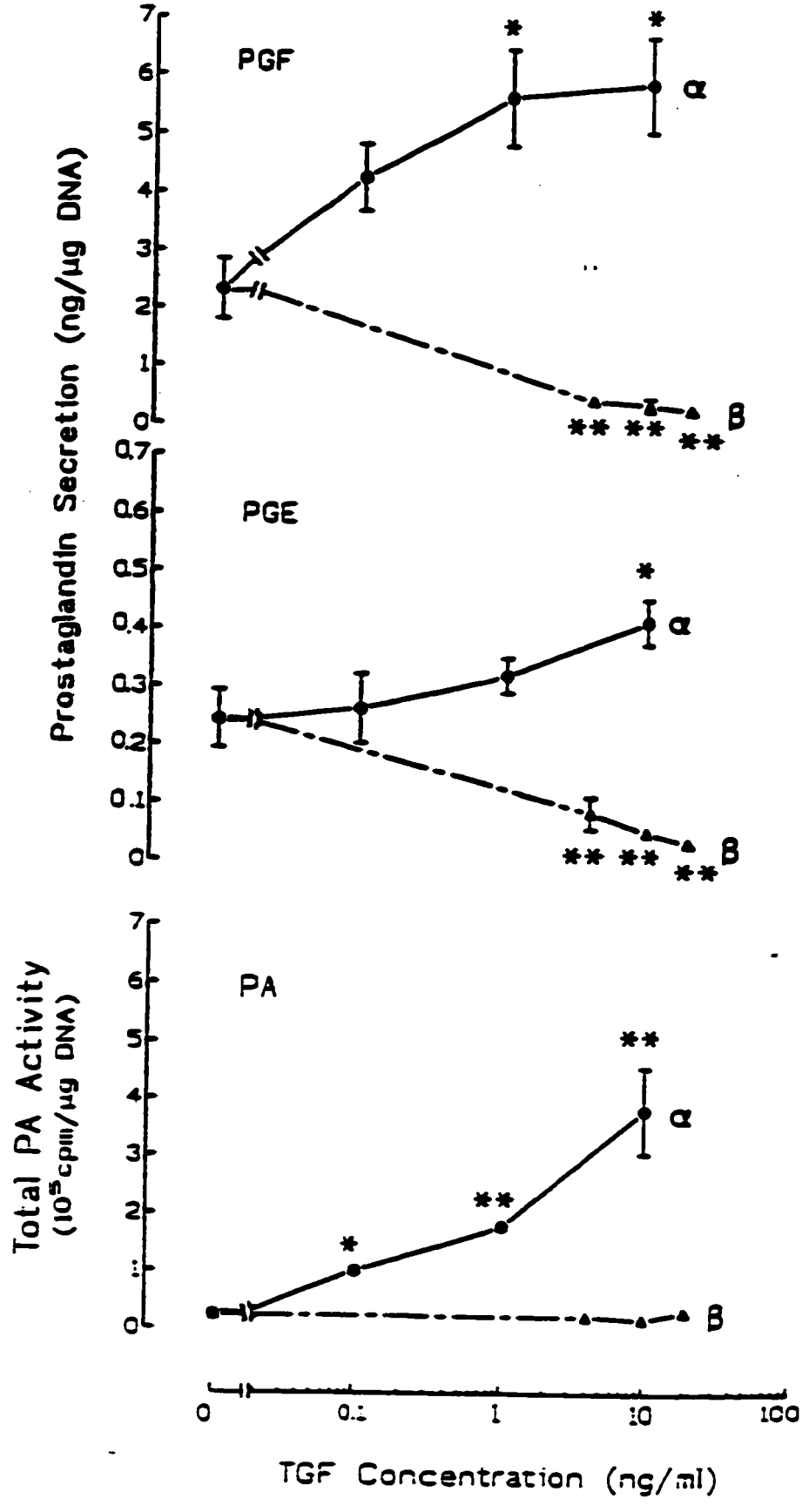

PGF
PGE
PA
Prostaglandin Secretion (ng/µg DNA)
Total PA Activity
(10^5 cpm/µg DNA)
α
β
TGF Concentration (ng/ml)
0
0.1
1
10
100

Figure 7: Influence of TGFα (10 ng/ml) and/or TGFß (20 ng/ml) on PGF (A), PGE (B) secretion and PA activity (C) in granulosa cells of F1, F3 and F5-6 follicles after 21 h culture. PA activity is the sum of PA activity in the cell (PAc) and medium (PAs) fractions. Values represent the means±SEM of five replicate experiments. ** $P<0.01$ (compared to control group); ++ $P<0.01$ (compared to TGFα group).

A
PGF Secretion
(ng/µg DNA)
B
PGE Secretion
(ng/µg DNA)
C
Total PA Activity
(10⁶cpm/µg DNA)
C α β α+β
F5-6
F3
F1

TABLE 3. Basal and TGFα-induced PA activity in cultured granulosa cells from different stages of follicular development.

	PA activity (10^3 cpm/μg DNA)*					
	F1		F3		F5-6	
	con	TFGα	con	TGFα	con	TGFα
PAc	36 ± 12	562 ± 216	229 ± 59	789 ± 303	714 ± 213	1068 ± 368
PAs	5 ± 1	93 ± 23	32 ± 9	247 ± 62	244 ± 55	1416 ± 523

Cell associated (PAc) and secreted (PAs) PA activity in the absence and presence of TGFα (10 ng/ml) in 21 h cultures of granulosa cells from F1, F3 and F5-6 follicles. Values are means + SEM of six experiments.

3. Time-dependent response of PG production to TGFα and TGFβ in vitro during follicular development

To examine the time course of action and interactions of TGFα and TGFβ on granulosa cell PG secretion, cells from F1 and F5-6 were cultured for 6, 12, 21 h in the absence and presence of TGFα and/or TGFß (Fig. 8 & 9). Whereas a stimulation of PGF secretion by TGFα was evident as early as 6 h, an inhibition of basal and TGFα-induced PGF secretion by TGFß was not observed until 12 and 21 h of culture, respectively, for both F1 and F5-6 (Fig. 8). On the contrary, PGE secretion by F1 cells, though significantly increased by TGFα throughout the culture period, was suppressed by TGFβ by 6 h (Fig. 9). However, inhibition of basal PGE secretion by TGFβ was only observed within 21h of culture. Likewise, there was no statistically significant effect of the growth factors on the secretion of PG by F5-6 granulosa cells until after 12 h of culture, when TGFα was stimulatory and TGFβ inhibitory.

To further clarify the relationship between PG secretion and PA activity in response to TGFα stimulation, granulosa cells from F1 and F5-6 follicles were cultured in the presence of TGFα (10ng/ml) and a cyclooxygenase inhibitor, indomethacin (0-0.5 μM). Although indomethacin markedly inhibited TGFα-induced PG secretion, it failed to significantly affect the TGFα-stimulated PA activity (Fig. 10).

Figure 8: Time course study of the effects of TGFα (10ng/ml) and/or TGFß (20ng/ml) on PGF secretion by granulosa cells from F1 and F5-6 follicles during 6, 12 and 21 h culture. Values represent means±SEM of three replicate experiments. * $P<0.05$, ** $P<0.01$ (compared to control group); + $P<0.05$, ++ $P<0.01$ (compared to TGFα group).

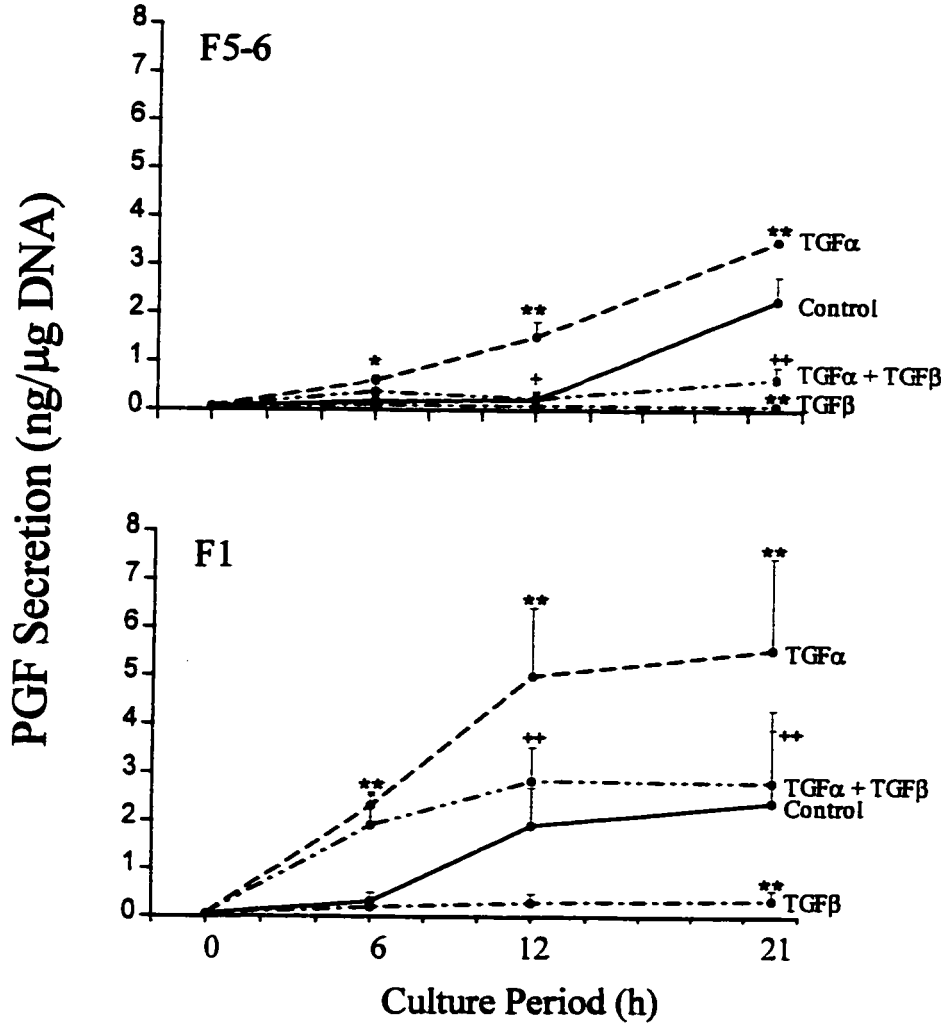

F5-6
F1
PGF Secretion (ng/μg DNA)
Culture Period (h)
TGFα
Control
TGFα + TGFβ
TGFβ
0
1
2
3
4
5
6
7
8
0
6
12
21

Figure 9: Temporal effects of TGFα (10ng/ml) and/or TGFß (20ng/ml) on PGE secretion by F1 and F5-6 granulosa cells during 6, 12 and 21 h culture. Values represent the means±SEM of three replicate experiments. * $P<0.05$, ** $P<0.01$ (compared to control group); + $P<0.05$, ++ $P<0.01$ (compared to TGFα group).

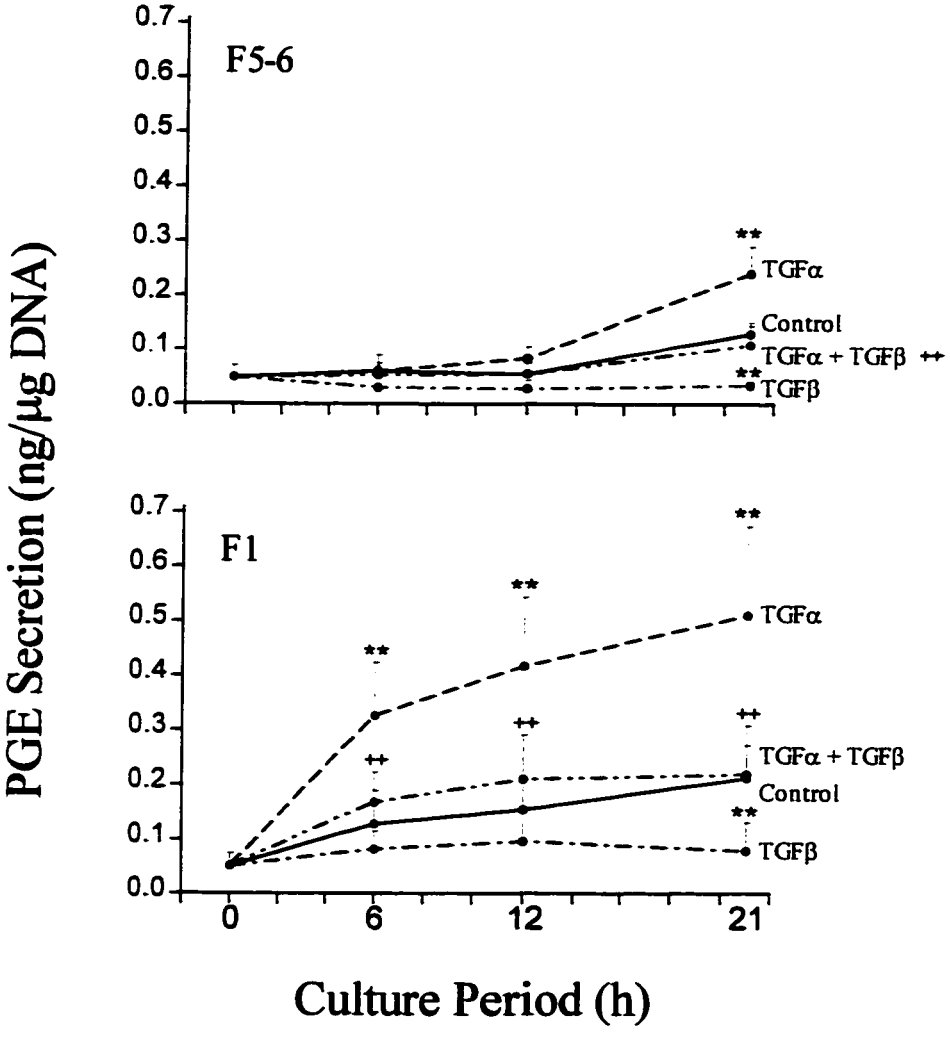
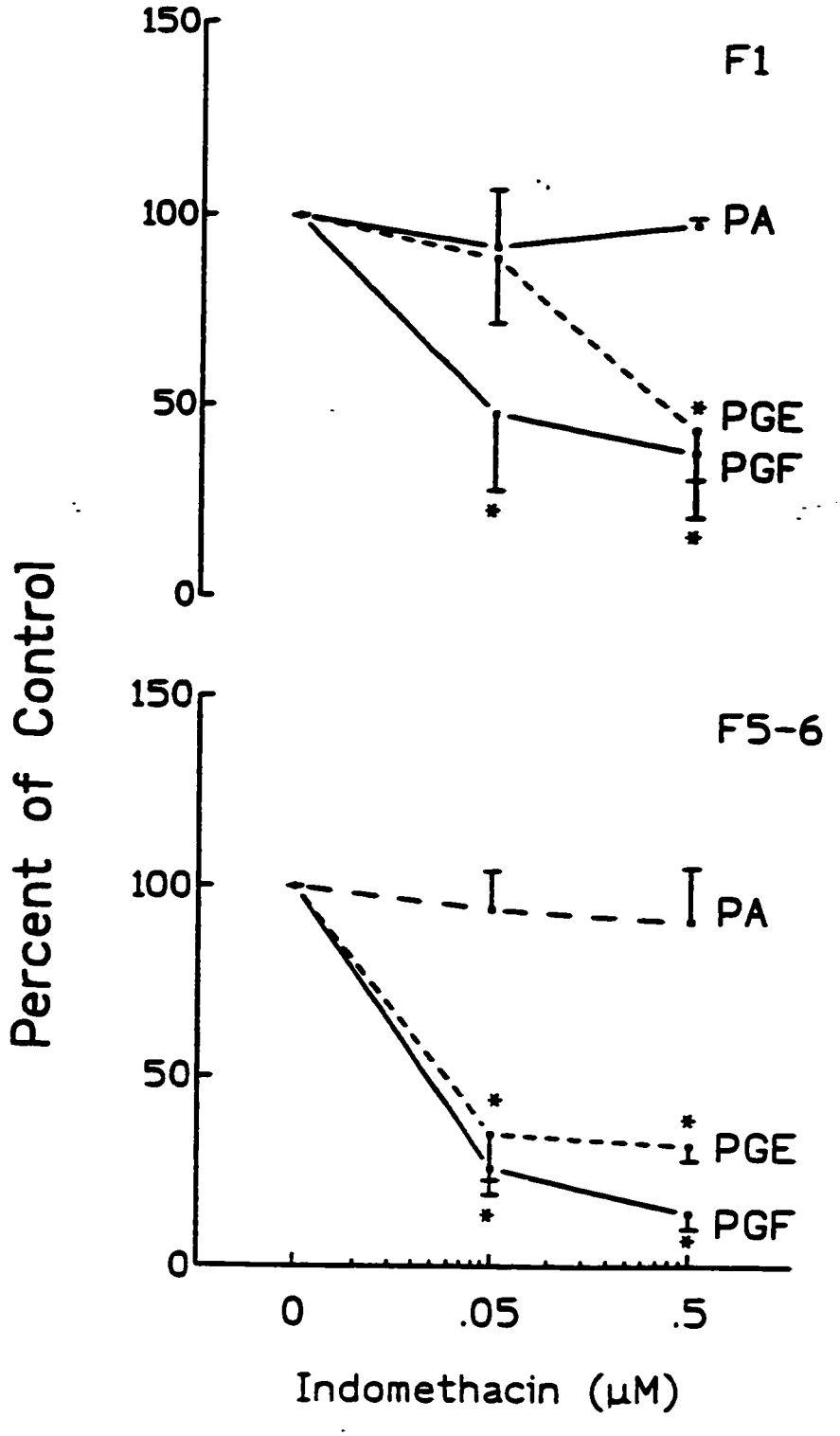

F5-6
F1
PGE Secretion (ng/μg DNA)
Culture Period (h)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
6
12
21
TGFα
Control
TGFα + TGFβ
TGFβ
**
++

Figure 10: Differential effects of indomethacin on TGFα (10 ng/ml)-induced PG secretion and PA activity in granulosa cells from F1 and F5-6 during a 21 h culture period. Values are expressed as percent of TGFα-induced response in the absence of indomethacin and represent the means±SEM of four replicate experiments. In the absence of indomethacin, TGFα-induced PGF and PGE secretion and PA activity was 1.8±0.3 ng/μg DNA, 0.7±0.05 ng/μg DNA and 6.1±1.0 x 10^5cpm/μg DNA, respectively in F1, 1.2 ±0.1 ng/μg DNA, 0.6±0.1 ng/μg DNA and 7.5±0.3 x 10^5cpm/μg DNA, respectively in F5-6. * $P<0.05$ (compared to TGFα stimulation)


F1
150
100
50
0
PA
PGE
PGF
F5-6
150
100
50
0
PA
PGE
PGF
Percent of Control
0
.05
.5
Indomethacin (μM)

B. TGFα-induced granulosa cell DNA synthesis is mediated by PGs during follicular development

1. TGFα stimulated PG production and DNA synthesis in F1 granulosa cells

Consistent with our previous findings (Lafrance et al., 1993b, Li et al., 1994), [^{3}H]thymidine incorporation into DNA ($P<0.01$), PGE ($P<0.01$) and PGF ($P<0.01$) production in granulosa cells from the F1 follicle increased in a concentration-dependent manner in response to TGFα (0.1-10ng/ml). At 1 ng/ml, TGFα significantly stimulated PGE (168%), PGF (244%) production and DNA synthesis (310%) (Fig. 11).

2. TGFα-induced DNA synthesis suppressed by inhibitors of phospholipase A_2 and cyclooxygenase but not of lipoxygenase

The influence of PLA_2 inhibitors on TGFα-induced DNA synthesis in granulosa cells was investigated to determine whether metabolites of arachidonic acid are essential for TGFα-stimulated mitogenesis. 2-(p-Amylcinnamoyl)amino-4-chlorobenzoic acid (ONO-RS-82) significantly suppressed TGFα-stimulated PG production and DNA synthesis in granulosa cells in a concentration-dependent manner. The ID_{50} of ONO-RS-82 for secretion of PGE and PGF as well as for [^{3}H]thymidine incorporation into DNA were >5, approximately 0.2 and 0.8 μM for F1 and approximately 0.2, 0.1 and 0.4 μM for F5-6 granulosa cells, respectively (Fig. 12). Similarly, [^{3}H]thymidine incorporation into DNA induced by TGFα was also attenuated by other PLA_2 inhibitors, aristolochic acid and 7,7-dimethyleicosadienoic acid (DEDA), although the inhibition was to a different extent (Fig. 13).

Figure 11: Concentration-response studies of the influence of TGFα on PG production and [^{3}H]thymidine incorporation into DNA in F1 granulosa cells during an 18 h culture period. Values represent means±SEM of six (PGE secretion) or three (PGF secretion and [^{3}H] thymidine incorporation) experiments. * $P<0.05$; ** $P<0.01$ (compared to control)

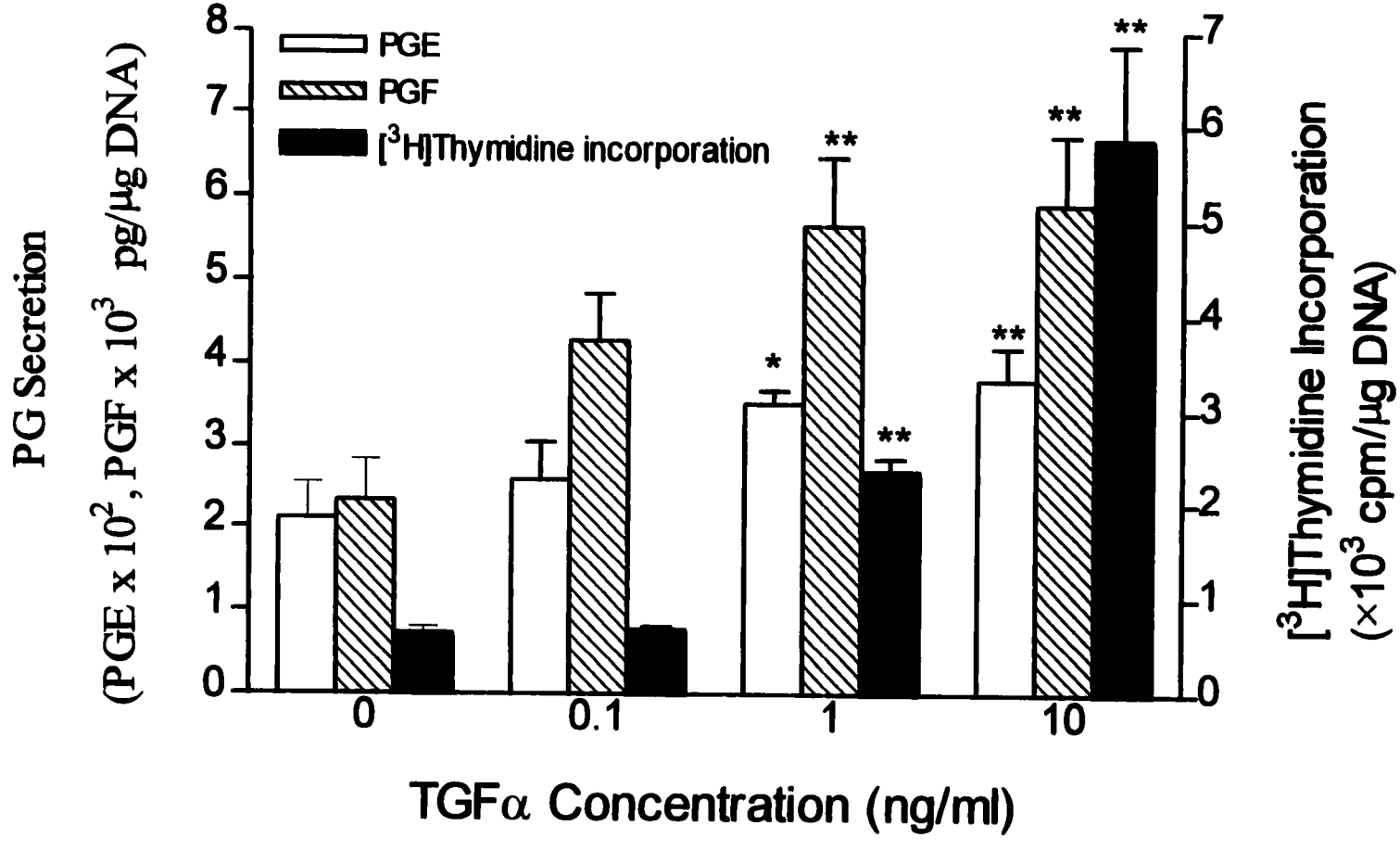

PG Secretion
(PGE x 10^2, PGF x 10^3 pg/μg DNA)
PGE
PGF
[^{3}H]Thymidine incorporation
[^{3}H]Thymidine Incorporation
(×10^3 cpm/μg DNA)
TGFα Concentration (ng/ml)
0
0.1
1
10
*
**

Figure 12: Concentration-dependent suppression of TGFα -induced PG production and [^{3}H]thymidine incorporation in granulosa cells from F1 and F5-6 follicles by the PLA_2 inhibitor ONO-RS-82 (0.1 - 5 μM) during 18 h of culture. TGFα (10 ng/ml)-induced PGF and PGE production were 2246 ± 646 pg/μg DNA and 729 ± 418 pg/μg DNA, respectively, for F1 and 1437 ± 649 pg/μg DNA and 178 ± 45 pg/μg DNA, respectively, for F5-6 granulosa cells. TGFα-induced [^{3}H]thymidine incorporation was 7679 ± 2345 cpm/μg DNA and 15145 ± 3405 cpm/μg DNA in F1 and F5-6 cells, respectively. Values represent means ± SEM of three to four experiments. * $P<0.05$; ** $P<0.01$ (compared to TGFα stimulation)

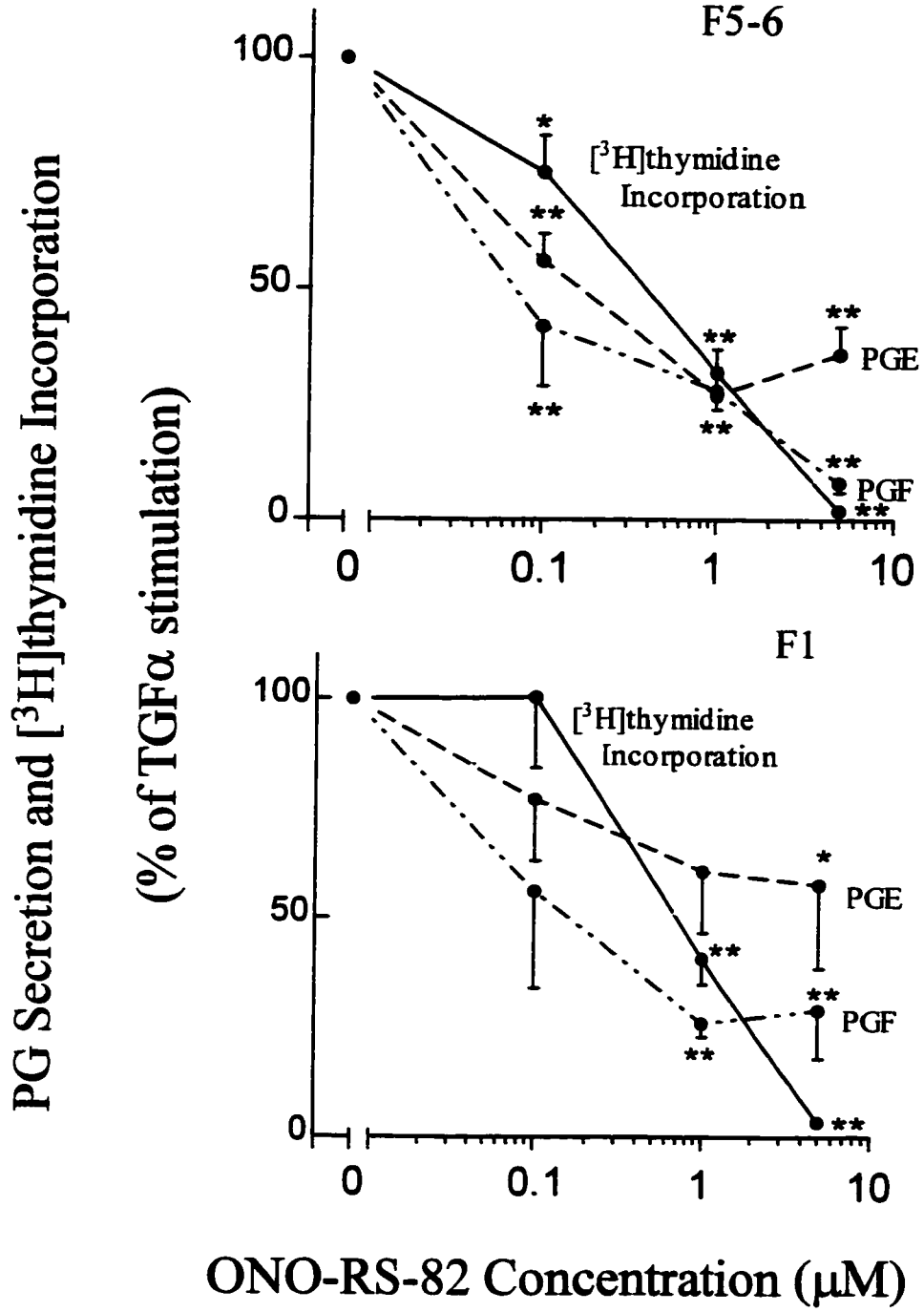
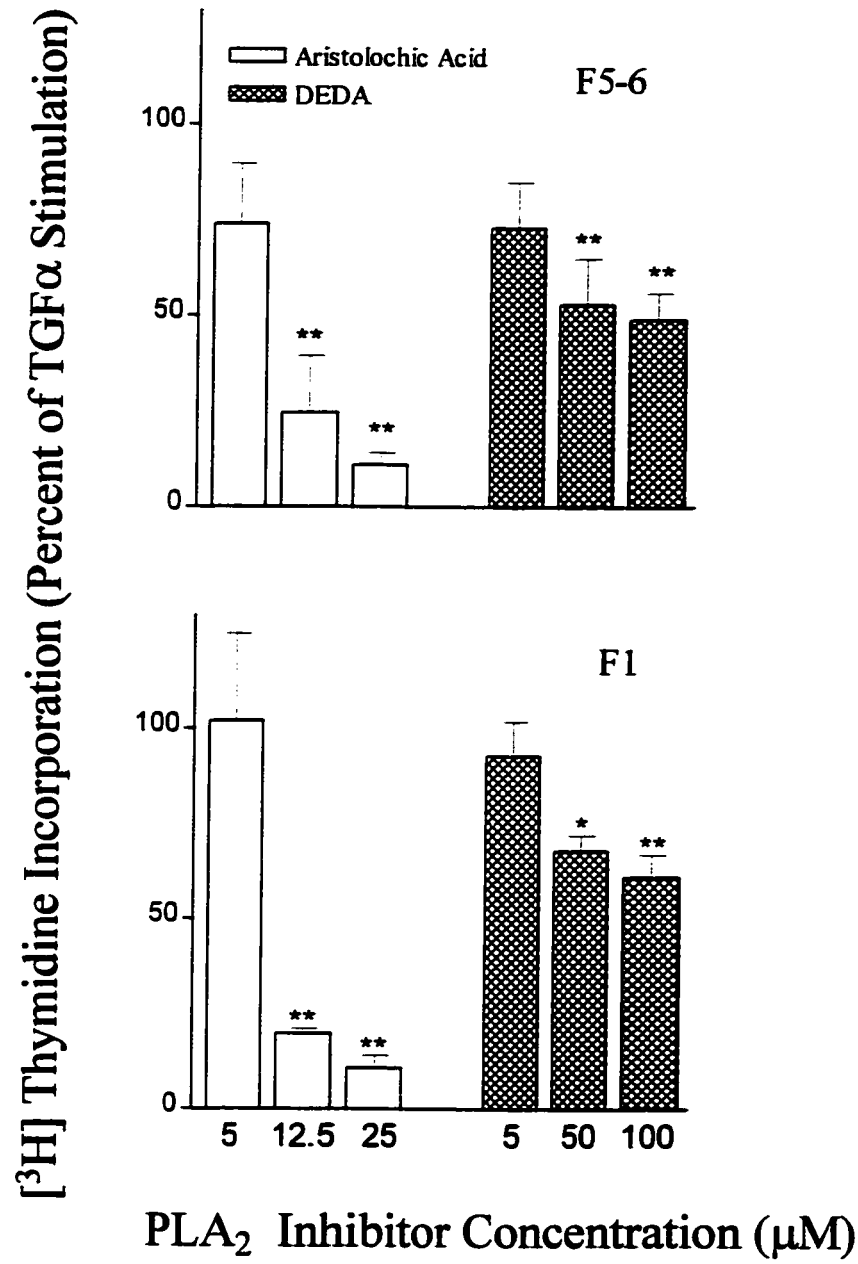

F5-6
100
50
0
[3H]thymidine Incorporation
PGE
PGF
0
0.1
1
10
F1
100
50
0
[3H]thymidine Incorporation
PGE
PGF
0
0.1
1
10
PG Secretion and [3H]thymidine Incorporation (% of TGFα stimulation)
ONO-RS-82 Concentration (μM)

Figure 13: Effect of the PLA_2 inhibitors aristolochic acid (5-25 μM) and DEDA (5-100 μM) on TGFα (10 ng/ml)-induced [^{3}H]thymidine incorporation in F1 and F5-6 granulosa cells during 18 h of culture. TGFα-induced thymidine incorporation were 14707±4516 and 17721±2751 cpm/μg DNA in F1 and F5-6 granulosa cells, respectively. Values are means±SEM of three experiments. ** $P<0.01$, *$P<0.05$ (compared toTGFα stimulation)


Aristolochic Acid
DEDA
F5-6
F1
100
50
0
**
*
5
12.5
25
50
[3H] Thymidine Incorporation (Percent of TGFα Stimulation)
PLA2 Inhibitor Concentration (μM)

To further investigate which arachidonic acid metabolite(s) might be involved in the regulation of DNA synthesis by TGFα, the influence of inhibitors of both cyclooxygenase and lipoxygenase pathways on [^{3}H]thymidine incorporation into DNA in granulosa cells cultured in the presence of the growth factor was examined. Addition of the cyclooxygenase inhibitors, naproxen and ibuprofen, to the culture medium diminished the TGFα-induced PG production (Fig. 14) and DNA synthesis (Fig. 15) in a concentration-dependent manner. Although indomethacin (0.05-5 μM) markedly inhibited PG production in granulosa cells from both F1 ($P<0.01$) and F5-6 ($P<0.01$) follicles, it failed to suppress the mitogenic response to TGFα and appeared to be slightly stimulatory to the response (Fig. 15). To determine if the failure of indomethacin to suppress TGFα-induced [^{3}H]thymidine incorporation into DNA was due to an unexpected effect of the inhibitor on DNA synthesis independent of its influence on PG production, granulosa cells were cultured with the cyclooxygenase inhibitor (0.05-5μM) in the presence of PGE_2 (5 μM) and a submaximal stimulatory concentration of TGFα (1 ng/ml). Indomethacin significantly ($P<0.05$) augmented [^{3}H]thymidine incorporation in granulosa cells stimulated by TGFα and PGE_2 (Fig. 16).

The lipoxygenase inhibitor MK-886 (0.1-2 μM), at concentrations reported to inhibit leukotriene production (Glasgow et al., 1992; Cowlen and Eling, 1992), failed to significantly affect the stimulation of granulosa cell DNA synthesis by TGFα irrespective of the stage of follicular development (Fig. 17).

Figure 14: Effects of cyclooxygenase inhibitors on TGFα (10 ng/ml) -induced PGF and PGE secretion in granulosa cells from F1 and F5-6 follicles. Values represent means±SEM of three experiments. * $P<0.05$; ** $P<0.01$ (compared to TGFα stimulation)

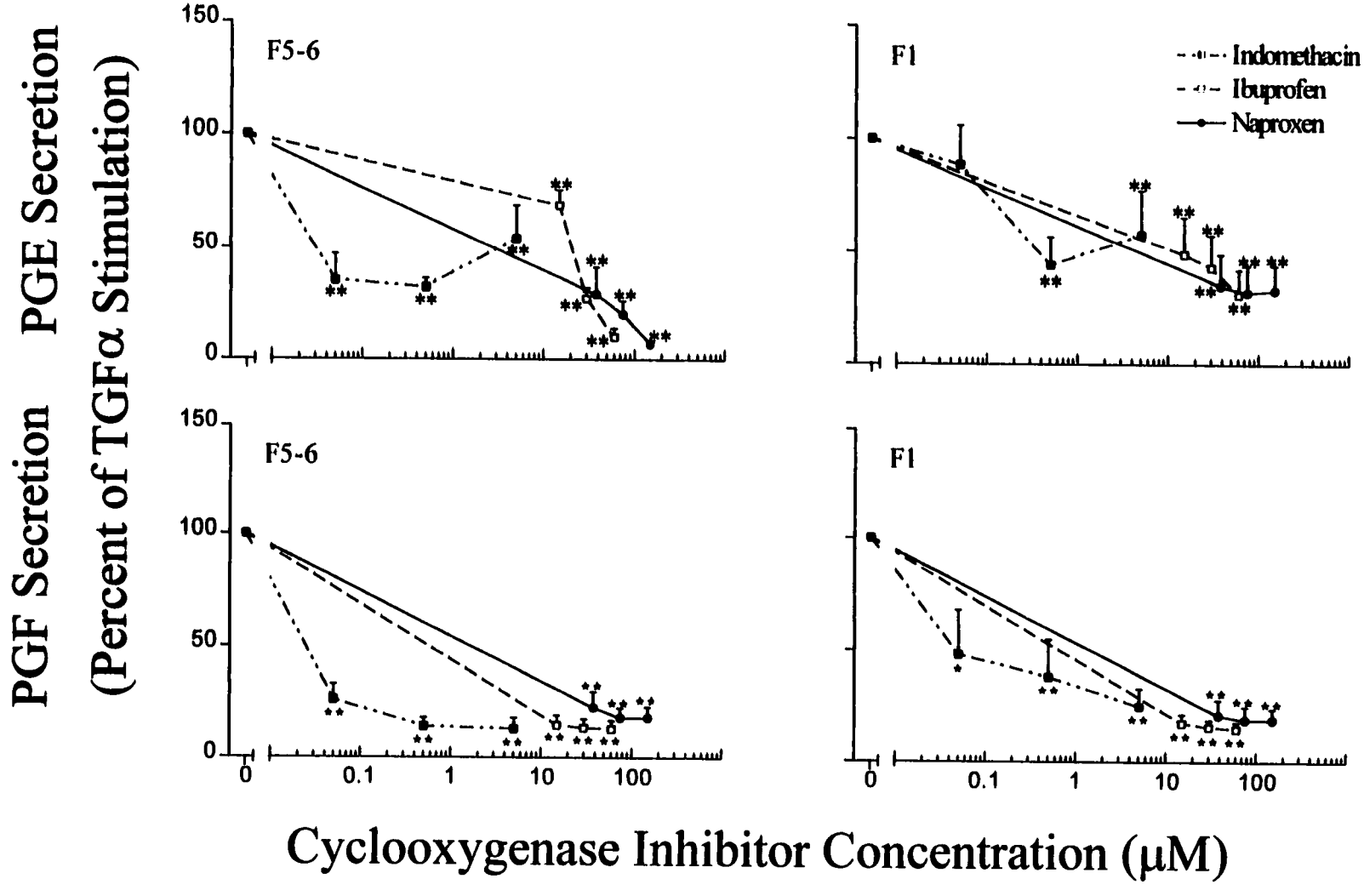

PGF Secretion PGE Secretion
(Percent of TGFα Stimulation)
F5-6
F1
Indomethacin
Ibuprofen
Naproxen
150
100
50
0
0.1
1
10
100
Cyclooxygenase Inhibitor Concentration (μM)

Figure 15: Effects of cyclooxygenase inhibitors on TGFα (10 ng/ml)-induced [^{3}H]thymidine incorporation in granulosa cells from F1 and F5-6 follicles. Values represent means ± SEM of four experiments. * P<0.05; **P<0.01 (compared to TGFα stimulation)

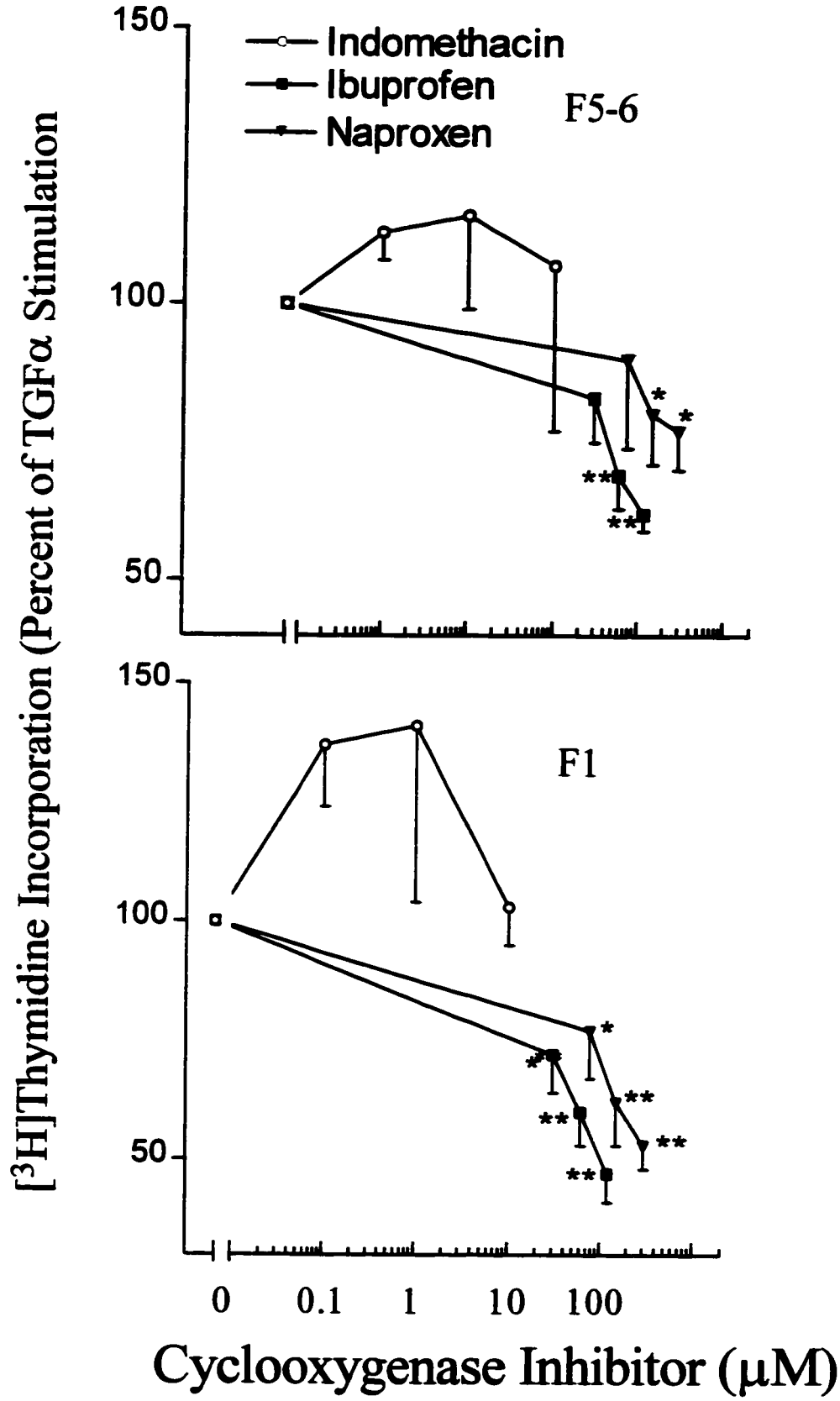

150
100
50
Indomethacin
Ibuprofen
Naproxen
F5-6
F1
[3H]Thymidine Incorporation (Percent of TGFα Stimulation
0
0.1
1
10
100
Cyclooxygenase Inhibitor (μM)

Figure 16: Stimulatory effect of indomethacin on PGE_2 (5μM)-enhanced [^{3}H]thymidine incorporation in F1 and F5-6 granulosa cells in the presence of TGFα (1 ng/ml). Values represent means±SEM of four experiments. * $P<0.05$ (compared to the TGFα plus PGE_2 stimulation group)

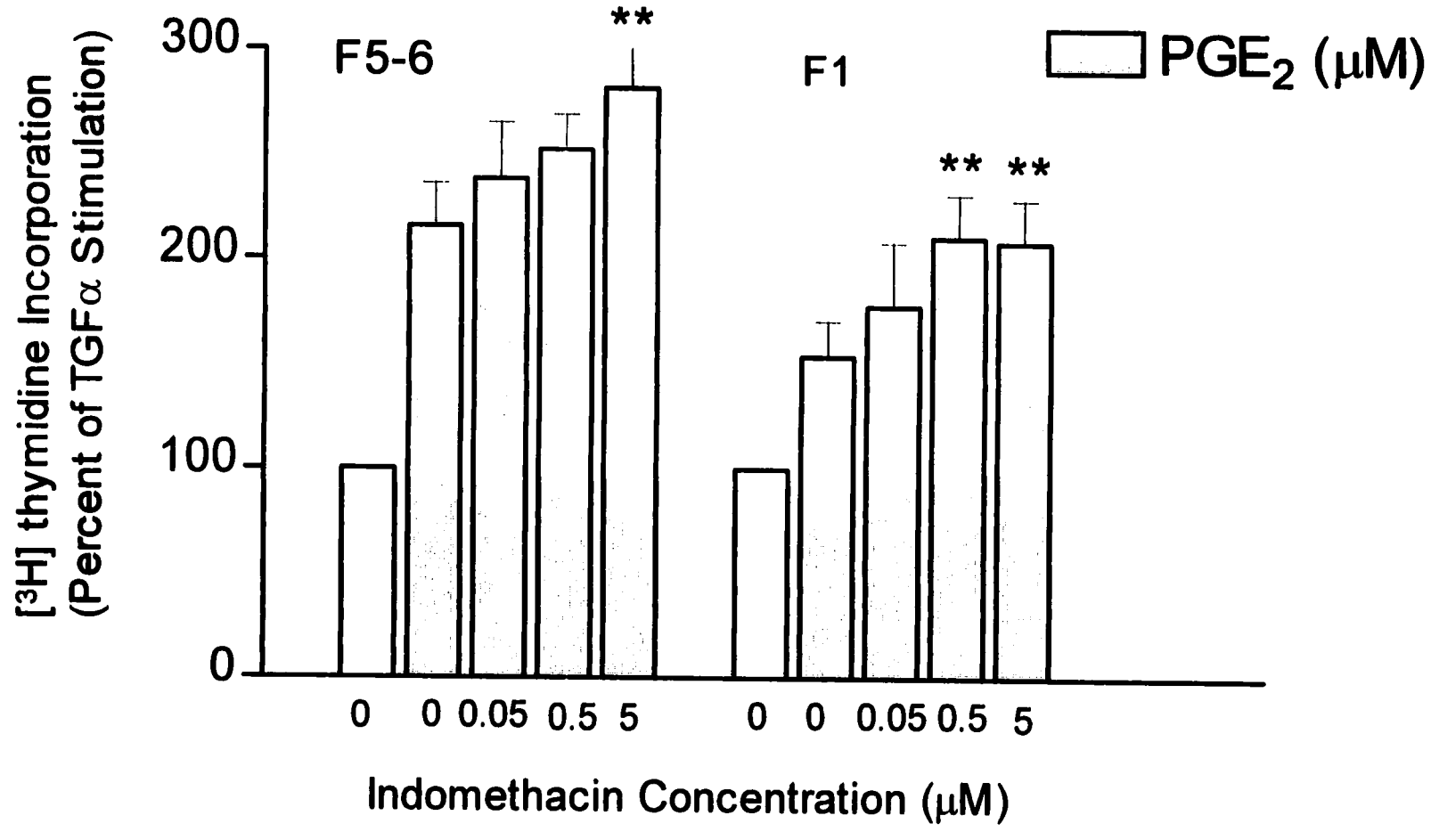
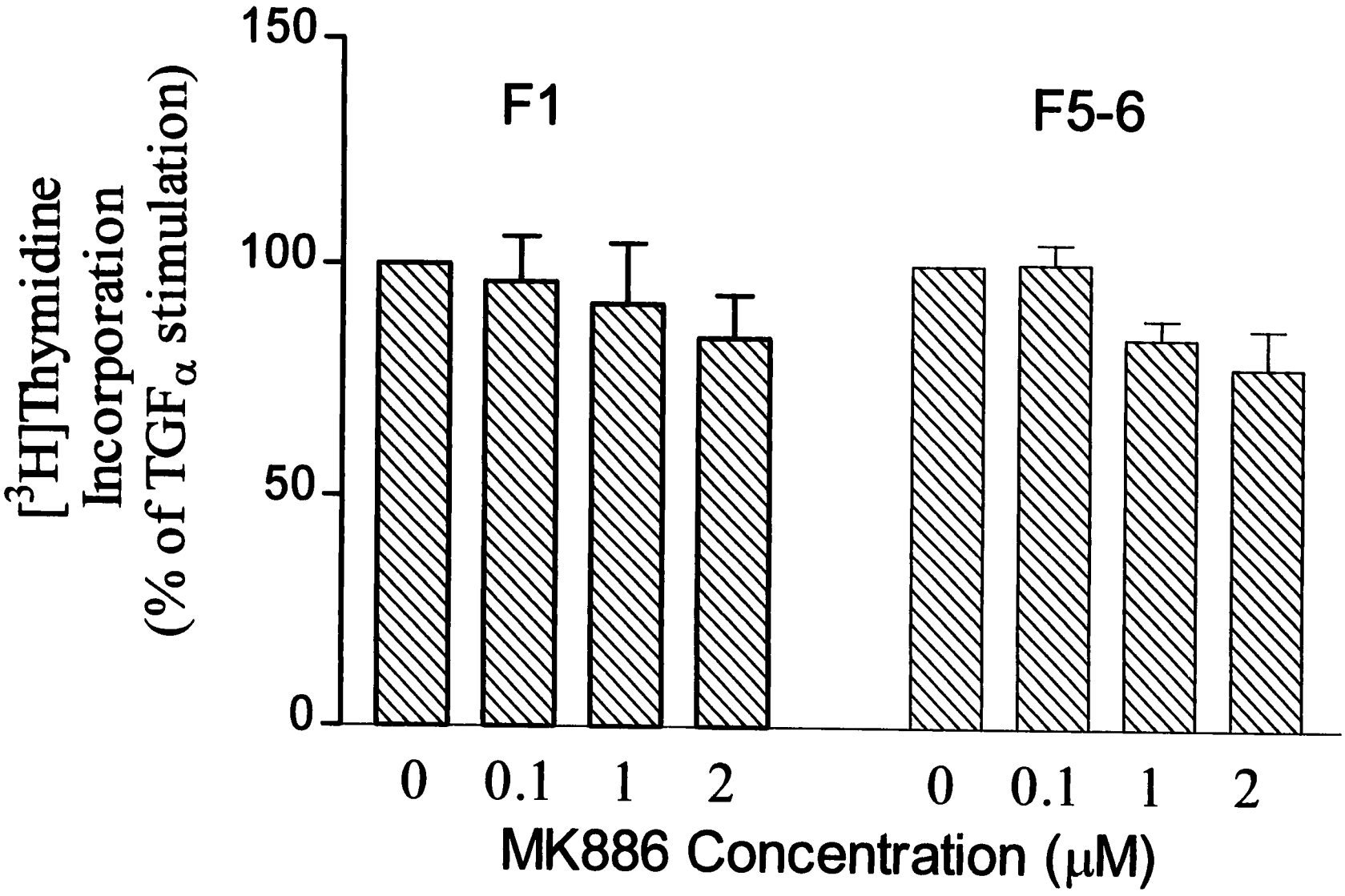

[3H] thymidine Incorporation
(Percent of TGFα Stimulation)
300
200
100
0
F5-6
F1
PGE2 (μM)
**
**
**
0
0
0.05
0.5
5
0
0
0.05
0.5
5
Indomethacin Concentration (μM)

Figure 17: Effect of lipoxygenase inhibitor on TGFα (10 ng/ml)-induced [^{3}H]thymidine incorporation in granulosa cells from F1 and F5-6 follicles. Values represent means±SEM of three experiments


150
100
50
0
F1
F5-6
[3H]Thymidine Incorporation (% of TGFα stimulation)
0
0.1
1
2
0
0.1
1
2
MK886 Concentration (μM)

3. PGE_2 attenuated the inhibition of TGFα-induced DNA synthesis by PLA_2 inhibitor

If prostaglandin formation is indeed an obligatory step in TGFα-induced DNA synthesis, it can be expected that the suppression of DNA synthesis by PLA_2 inhibitors could be reversed by exogenous PGs. As indicated in Figure 18, PGE_2 was capable of overcoming the inhibition of ONO-RS-82 (1 μM) on TGFα-stimulated DNA synthesis in granulosa cells. Although [^{3}H]thymidine incorporation into DNA was significantly increased by PGE_2 in granulosa cells from F5-6 and F1 follicles, the magnitude of stimulation was greater in cells from the less mature follicles ($p<0.01$). In addition, a lower concentration of PGE_2 appeared to be needed to elicit the maximal reversal of the inhibition in the proliferatively more active granulosa cells (F5-6). DNA synthesis was maximal when the cells were cultured in medium containing 0.5 μM (F5-6) and 5.0 μM (F1) PGE_2, respectively (Fig. 18).

4. Action of TGFα on DNA synthesis mimicked and potentiated by exogenous PGs

The addition of $PGF_{2\alpha}$ or PGE_2 alone to granulosa cell cultures resulted in a concentration- and follicular stage-dependent increase in DNA synthesis ($PGF_{2\alpha}$ stimulation was significantly only in F1 granulosa cells, Fig. 19), although these responses were considerably lower than that observed in the presence of TGFα alone (<100% vs 900%; compared to Fig. 11). Whereas maximal stimulation was achieved at 0.5 μM of PGE_2 in F5-6 granulosa cells, a higher concentration (5 μM) was required in the highly differentiated cells (F1; Fig. 19). However, the addition of a submaximal stimulatory concentration of TGFα (1ng/ml) markedly enhanced the responsiveness of the F5-6 granulosa cells to both PGE_2 (50 μM: 225% vs 60%) and $PGF_{2\alpha}$ (50 μM: 110% vs 30%). F1 granulosa cells cultured in the presence of TGFα responded with significant increases in [^{3}H]thymidine incorporation at

lower concentrations of PGs (one tenth) than those in the absenceof the growth factor (Fig. 19; Fig. 20). Further experiments have also demonstrated that both TGFα and PG-induced [^{3}H]thymidine incorporation were inhibited dose-dependently by hydroxyurea, a DNA synthesis inhibitor, suggesting that the increases were mainly due to DNA synthesis (Fig. 21). A comparison of the specificity of PGs on TGFα-induced DNA synthesis indicates that PGE_1 and PGE_2 are more effective than $PGF_{2\alpha}$ in cells from both stages of follicular development, while $PGF_{1\alpha}$ was inhibitory at the same stages (Table 4).

5. Lack of effects of LPA, LPC and products of lipoxygenase pathway on DNA synthesis

PLA_2 cleaves membrane lipids to AA and LPC which is further metabolized to LPA. Since TGFα-induced DNA synthesis was significantly suppressed by PLA_2 inhibitors (Fig. 12 & 13), we have also examined whether LPC and LPA, in addition to PGs, are involved in this regulation. Neither LPC nor LPA (0.1-10 μM) significantly influenced basal, and PGE_2-induced [^{3}H]thymidine incorporation into DNA in granulosa cells from F1 and F5-6 ($P>0.05$; Fig. 22). To further determine if arachidonic acid metabolites of the lipoxygenase pathway are involved in TGFα-induced DNA synthesis, the influence of LTB_4 (0.02-1 μg/ml), LTC_4 (0.02-1μg/ml), LTD_4 (0.02-1μg/ml) and 5-HETE (0.2-4 μM) on [^{3}H]thymidine incorporation into DNA was examined. Irrespective of the stage of follicular maturation and presence of exogenous TGFα (1 ng/ml; data not shown), none of the eicosanoids studied significantly influenced the DNA synthetic capacity of granulosa cells *in vitro* ($P>0.05$; Fig. 23).

6. EP3 receptor expression was greater in granulosa cells from early stage of follicular development

To determine whether the greater DNA synthetic response of granulosa cells from F5-6 to PGs was due to the higher abundance of EP3 receptors at this stage of follicular

development, granulosa cells from F1, F3, F5-6 were isolated and proteins were extracted. Western blot analysis was performed, using antibodies against EP3 (a PGE receptor subtype). As shown in Figure 24, EP3 expression was highest in granulosa cells at early stages of follicular development and decreased with follicular maturation.

Figure 18: Attenuation of the inhibitory effect of ONO-RS-82 (ONO; 1 μM) on TGFα (10 ng/ml)-induced [^{3}H]thymidine incorporation in F1 and F5-6 granulosa cells by PGE_2 *in vitro*. Values represent means±SEM of four experiments. * $P<0.05$; ** $P<0.01$ (compared to cells treated only with ONO and TGFα)

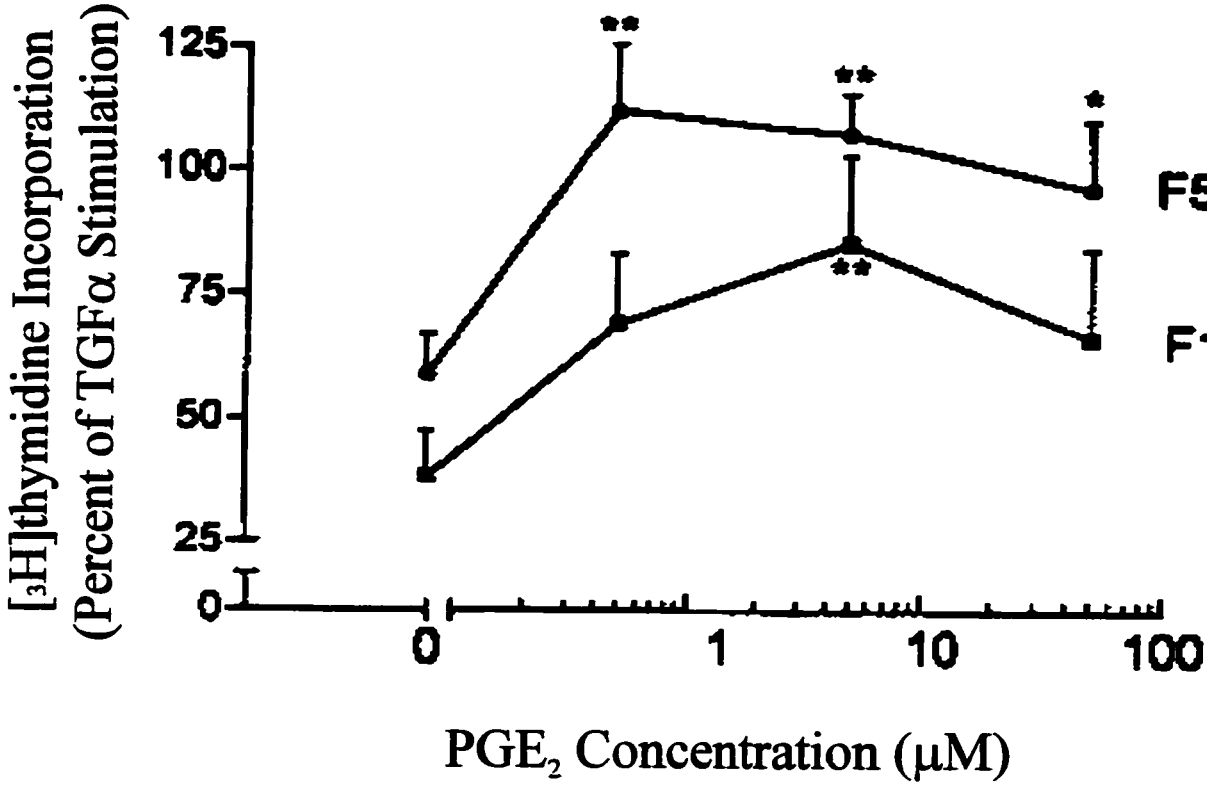

[3H]thymidine Incorporation
(Percent of TGFα Stimulation)
125
100
75
50
25
0
0
1
10
100
PGE2 Concentration (μM)
**
**
*
**
F5

Figure 19: The effects of PGE_2 and $PGF_{2\alpha}$ on DNA synthesis in F1 (—) and F5-6 (----) granulosa cells *in vitro*. Values are means ± SEM of three experiments. * $P<0.05$; ** $P<0.01$ (compared to control)

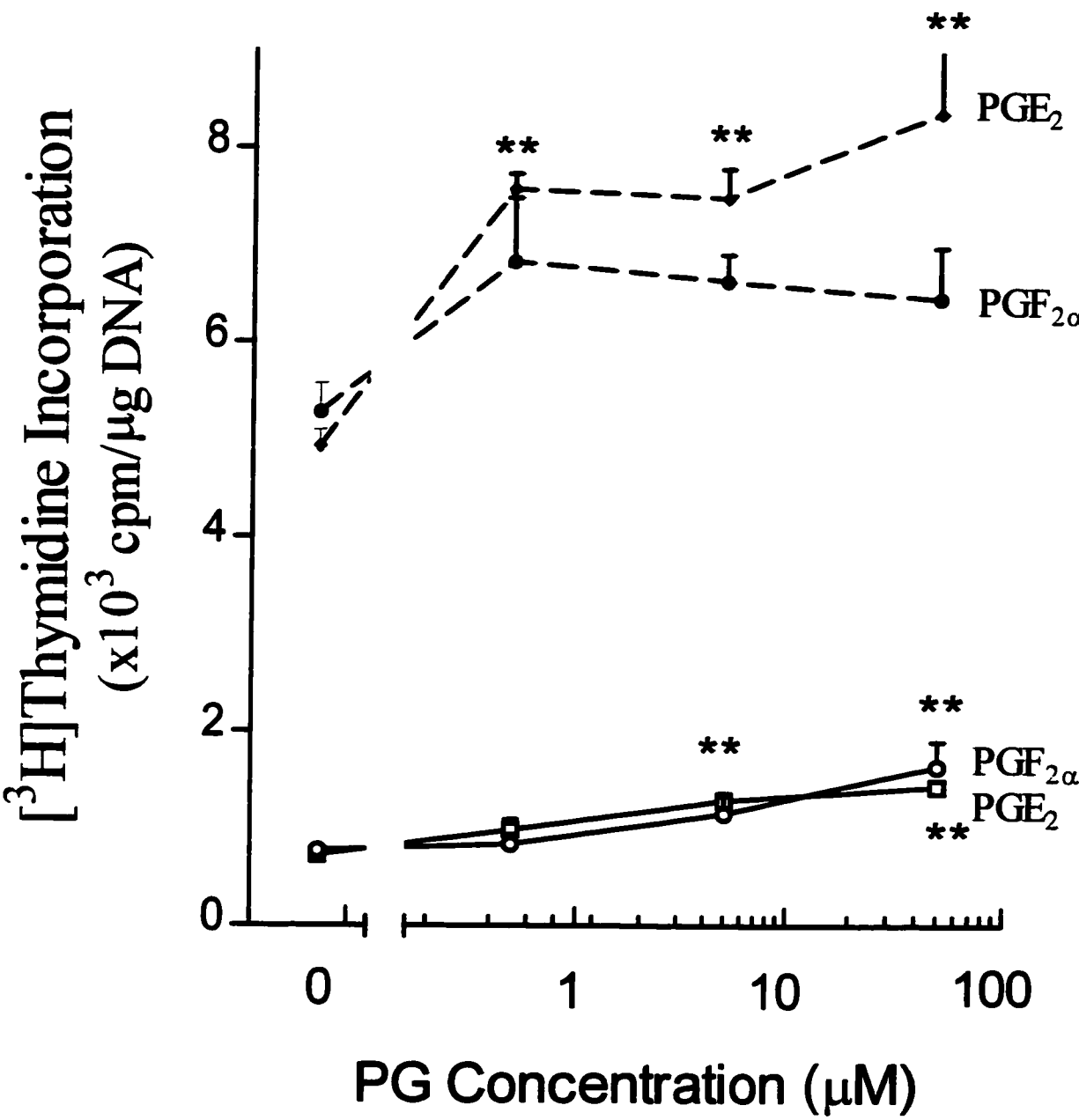

[3H]Thymidine Incorporation
(x10^3 cpm/µg DNA)
8
6
4
2
0
**
**
**
PGE2
PGF2α
**
**
**
PGF2α
PGE2
0
1
10
100
PG Concentration (µM)

Figure 20: Enhancement of TGFα-induced DNA synthesis in granulosa cells from F1 and F5-6 follicles by PGs. [^{3}H]thymidine incorporation in the presence of TGFα (1 ng/ml) alone in F1 and F5-6 granulosa cells were 10714±4393 cpm/μg DNA and 6258±850 cpm/μg DNA, respectively. Values are means ± SEM of four experiments. * $P<0.05$; ** $P<0.01$ (compared to TGFα stimulation)

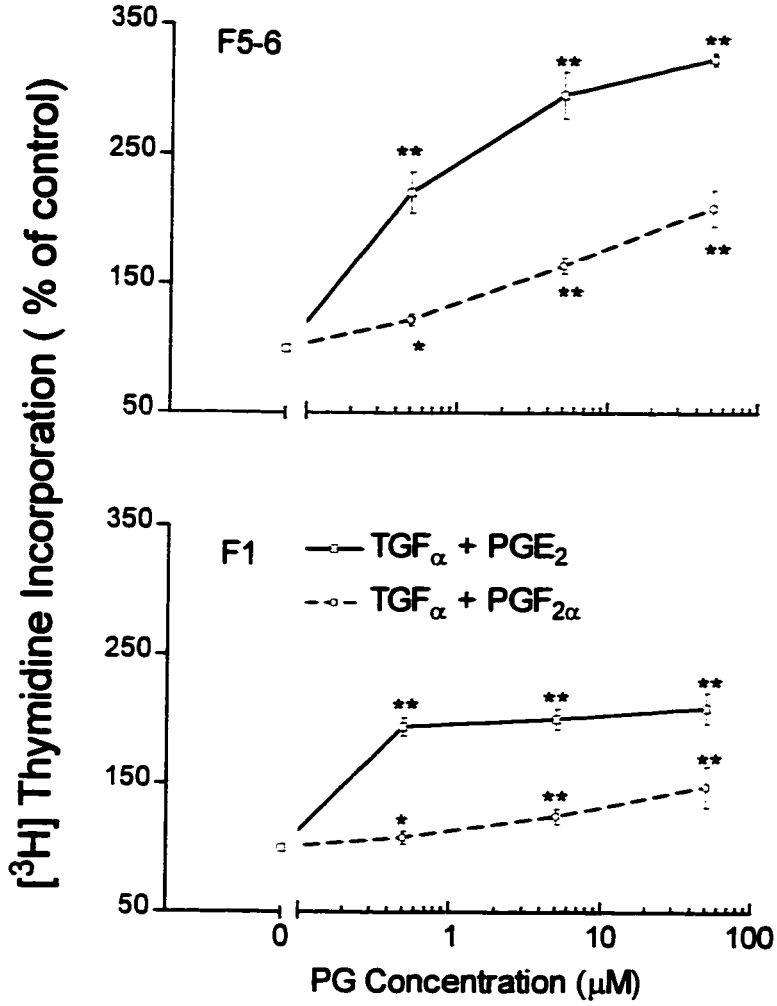
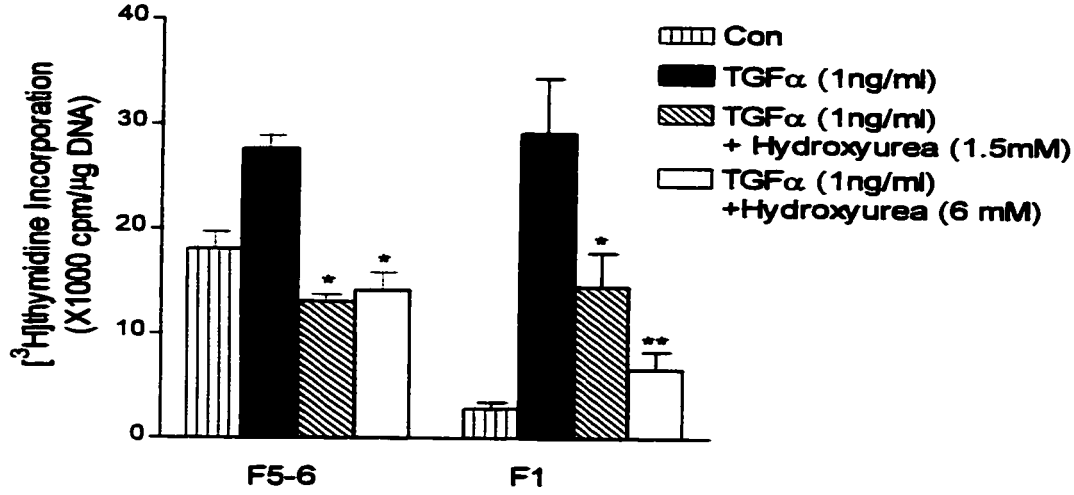

F5-6
F1
TGF$_\alpha$ + PGE$_2$
TGF$_\alpha$ + PGF$_{2\alpha}$
[^{3}H] Thymidine Incorporation (% of control)
PG Concentration (μM)
350
250
150
50
0
1
10
100
**
*

Figure 21: Dose-dependent inhibition of PG- and TGFα-induced [^{3}H]thymidine incorporation in granulosa cells by hydroxyurea. Values are means ± SEM of three experiments. * P<0.05; ** P<0.01 [compared to TGFα (*upper panel*) or TGFα + PGE_2 *(lower panel*) stimulation, respectively]


[3H]thymidine Incorporation (X1000 cpm/μg DNA)
40
30
20
10
0
F5-6
F1
Con
TGFα (1ng/ml)
TGFα (1ng/ml) + Hydroxyurea (1.5mM)
TGFα (1ng/ml) +Hydroxyurea (6 mM)

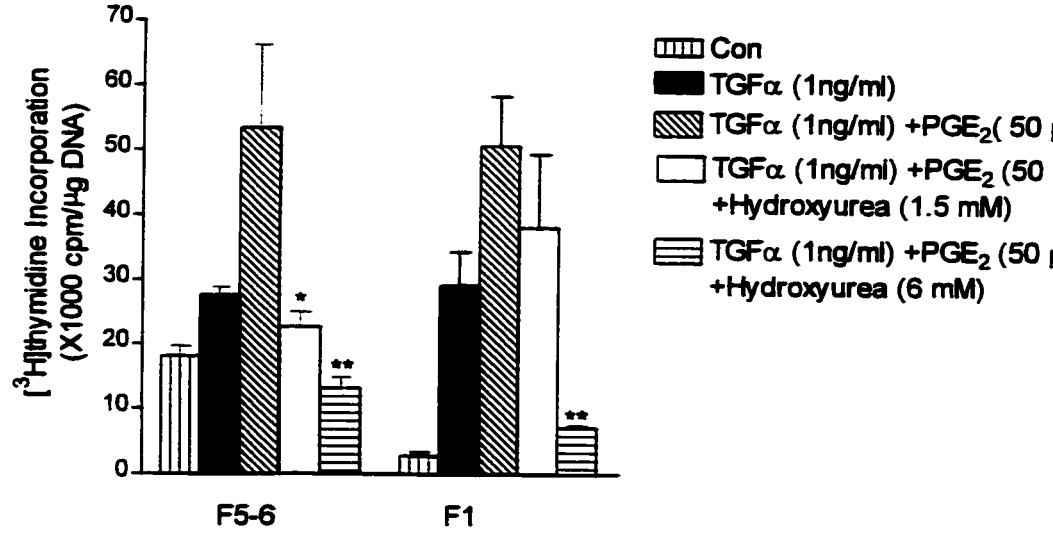

[3H]thymidine Incorporation (X1000 cpm/μg DNA)
70
60
50
40
30
20
10
0
F5-6
F1
Con
TGFα (1ng/ml)
TGFα (1ng/ml) +PGE2(50 μ
TGFα (1ng/ml) +PGE2 (50 μ +Hydroxyurea (1.5 mM)
TGFα (1ng/ml) +PGE2 (50 μ +Hydroxyurea (6 mM)

Table 4: **Potentiation of TGFα-induced DNA Synthesis in Granulosa Cells During Follicular Development: Specificity of PG Action.**

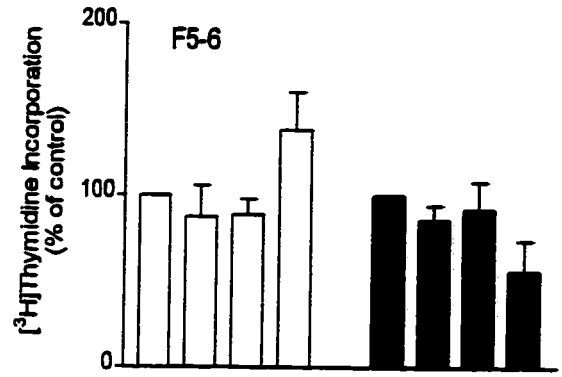
Treatment	[^{3}H]Thymidine Incorporation into DNA (% of TGFα stimulation)$^+$	
	F5-6	F1
TGFα (1ng/ml)	100	100
TGFα (1ng/ml) + PGE_1 (5μM)	290 ± 61 **	169 ± 16 *
TGFα (1ng/ml) + PGE_2 (5μM)	268 ± 62 **	200 ± 31 **
TGFα (1ng/ml) + $PGF_{1\alpha}$ (5μM)	63 ± 11 *	62 ± 8 *
TGFα (1ng/ml) + $PGF_{2\alpha}$ (5μM)	174 ± 28 *	115 ± 16

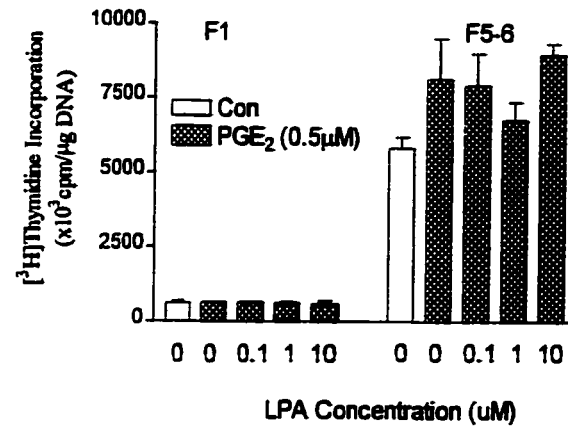
$^+$ [^{3}H]Thymidine incorporation into DNA in F5-6 and F1 granulosa cells in the presence of TGFα was 11539 ± 3548 cpm/μg DNA and 11511 ± 4311 cpm/μg DNA, respectively. Values represent means ± SEM of three experiments.

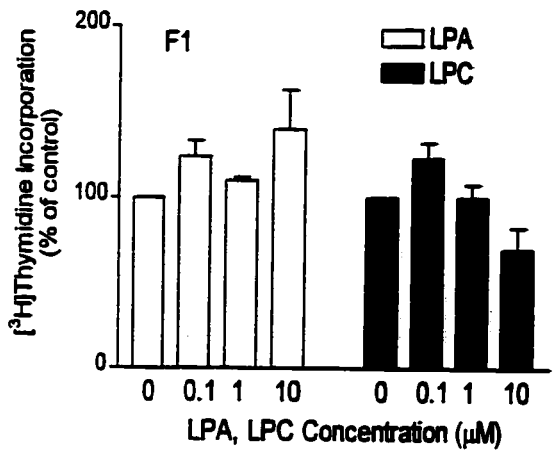
* $P<0.05$

** $P<0.01$

Figure 22: **Failure of LPA and LPC to influence basal and PGE_2-induced [^{3}H]thymidine incorporation in granulosa cells from F1 and F5-6 follicles *in vitro*. Values are means±SEM of three experiments.**


F5-6
[3H]Thymidine Incorporation (% of control)
200
100
0


F1
F5-6
Con
PGE2 (0.5μM)
[3H]Thymidine Incorporation (x10³cpm/μg DNA)
10000
7500
5000
2500
0
0 0 0.1 1 10 0 0 0.1 1 10
LPA Concentration (uM)


F1
LPA
LPC
[3H]Thymidine Incorporation (% of control)
200
100
0
0 0.1 1 10 0 0.1 1 10
LPA, LPC Concentration (μM)

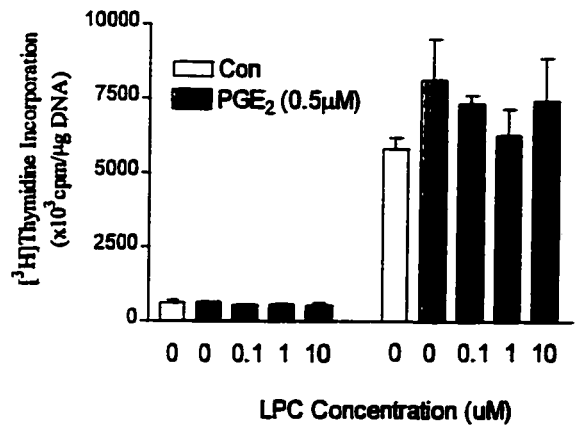

Con
PGE2 (0.5μM)
[3H]Thymidine Incorporation (x10³cpm/μg DNA)
10000
7500
5000
2500
0
0 0 0.1 1 10 0 0 0.1 1 10
LPC Concentration (uM)

Figure 23: Lack of effects of leukotrienes on basal and TGFα-induced [^{3}H]thymidine incorporation in granulosa cells from F1 and F5-6 follicles *in vitro*. Values are means±SEM of two to three experiments.

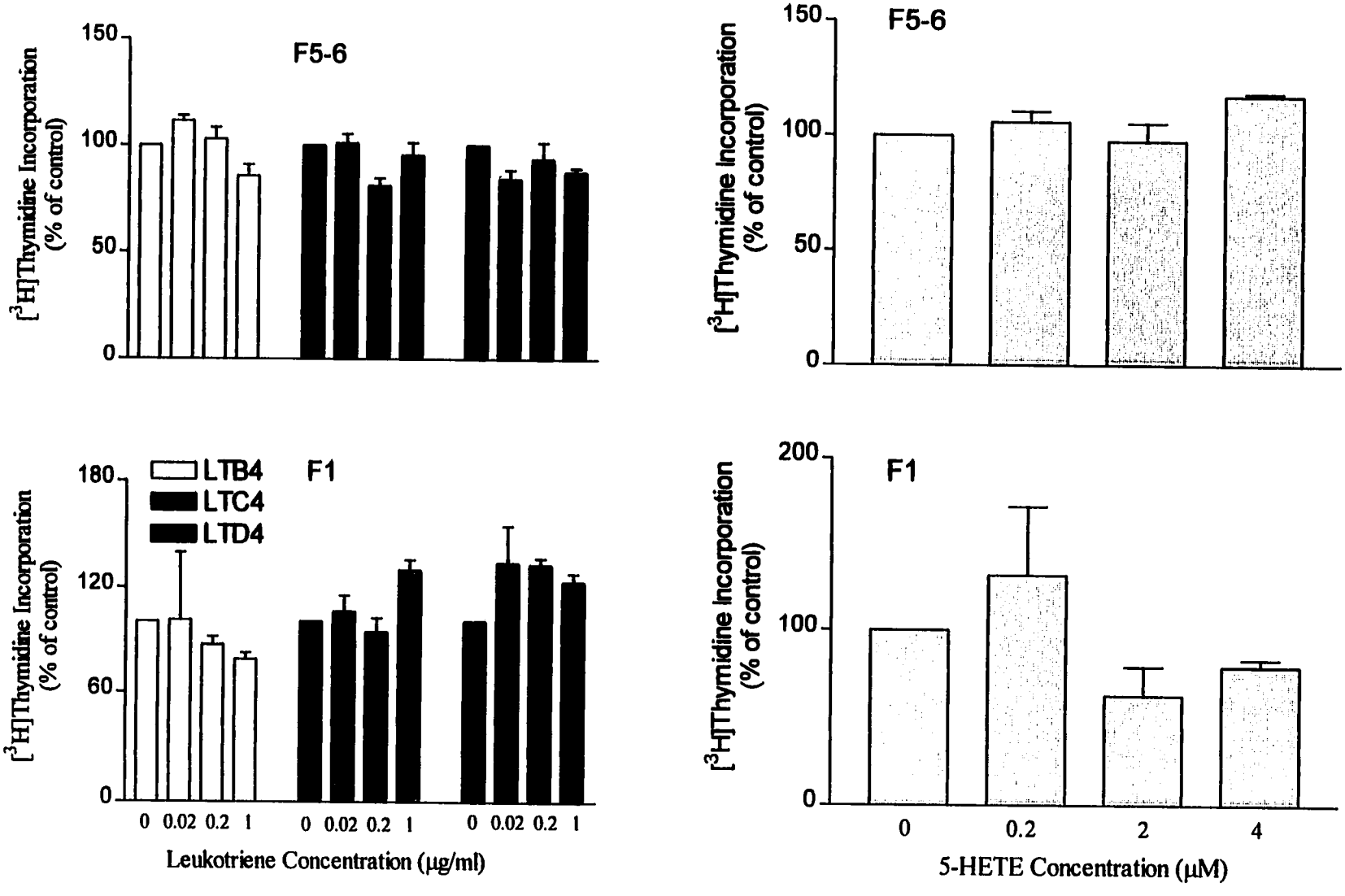
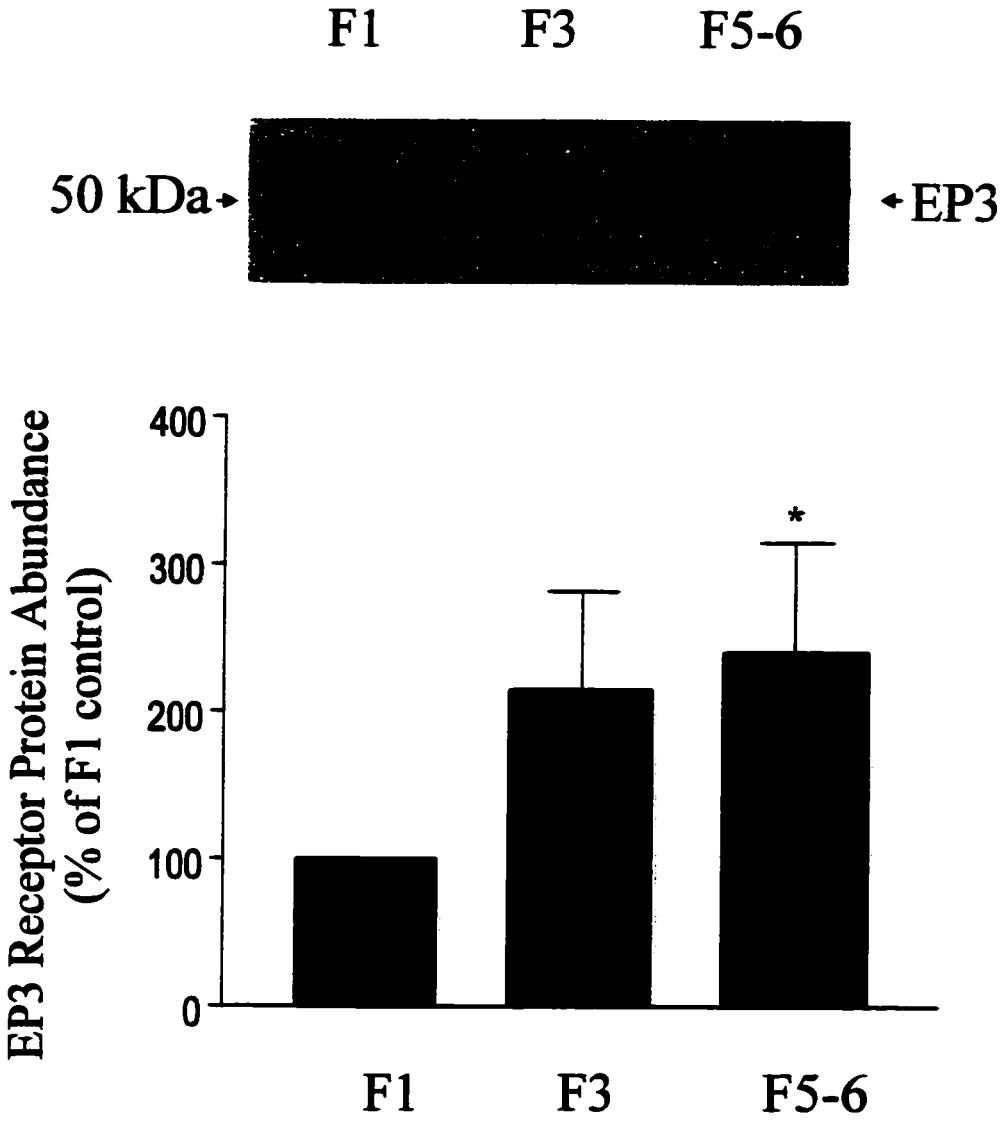

F5-6
[3H]Thymidine Incorporation (% of control)
150
100
50
0
F5-6
[3H]Thymidine Incorporation (% of control)
150
100
50
0
LTB4
LTC4
LTD4
F1
[3H]Thymidine Incorporation (% of control)
180
120
60
0
0 0.02 0.2 1 0 0.02 0.2 1 0 0.02 0.2 1
Leukotriene Concentration (µg/ml)
F1
[3H]Thymidine Incorporation (% of control)
200
100
0
0 0.2 2 4
5-HETE Concentration (µM)

Figure 24: EP3 receptor levels in granulosa cells during follicular development. Granulosa cells were isolated from different stages of follicular development and solubilized cell protein (100 μg/lane) was analyzed by Western blot, using an anti-EP3 antibody. Densitometric analysis of EP3 expression was performed using a Molecular Dynamics Phosphoimager. Upper panel shows a representative filter and lower panel shows the mean±SEM of three experiments. *$p<0.05$ (compared to F1)

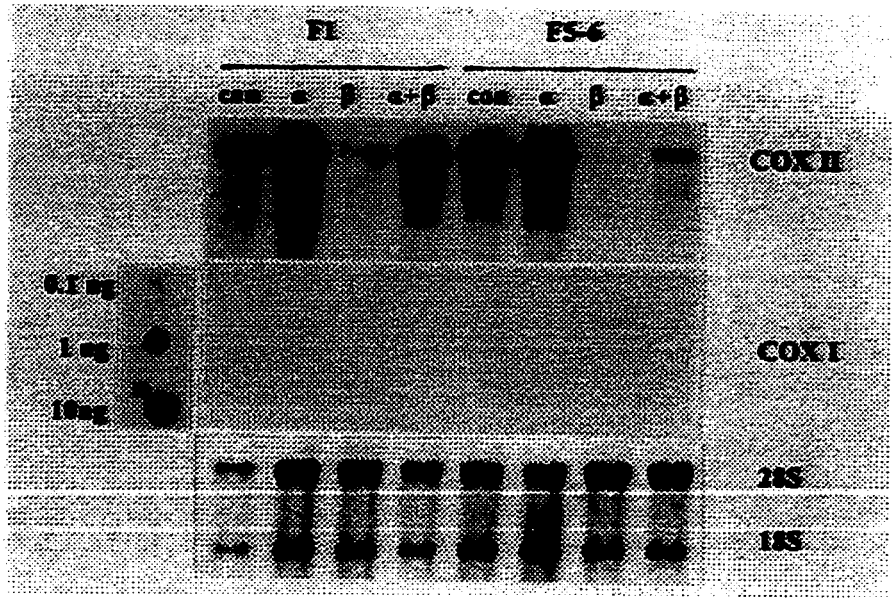

F1
F3
F5-6
50 kDa
EP3
EP3 Receptor Protein Abundance
(% of F1 control)
400
300
200
100
0
*
F1
F3
F5-6

C. **COX II is important in the signalling cascade for TGFα in the regulation of granulosa cell PG production and thus DNA synthesis during follicular development.**

1. Selective regulation of COX II mRNA in hen granulosa cells by transforming growth factors

To determine whether PG production is regulated by growth factors at the transcriptional level, granulosa cells from F1 and F5-6 follicles were cultured for 8 h in the absence or presence of TGFα and TGFβ. Northern analysis indicates the presence of COX II mRNA in cultures of granulosa cells from both F1 and F5-6. Whereas TGFα increased the abundance of COX II mRNA, both basal and TGFα-induced changes in the transcript level were suppressed by TGFβ (Fig. 25). In addition, the regulation of COX II mRNA abundance by the growth factors was dependent on follicular maturation. Although there was no significant difference in the magnitude of the maximal response of the cells to TGFα (approximately 300%) with follicular development (ANOVA, $p>0.05$), the temporal response pattern of COX II mRNA was markedly different. While the time required by the growth factor to elicit the maximal response was significantly shorter in cells from F1 than F5-6 (4 h vs. 8 h), the transient nature of this response was evident after 12 h in the cells from the more mature follicles (F1) [Fig. 26]. However, the suppression of both basal and TGFα-induced changes in the transcript level by TGFβ was greater in cells from less mature follicles (i.e. TGFα-stimulated response to level below untreated control; Fig. 25), as demonstrated by a statistically significant interaction between follicular stage and TGFβ action (ANOVA, $p<0.001$). Interestingly, the relative abundance of the transcript between

Figure 25: Effect of growth factors on granulosa cell mRNA abundance of COX isozymes. Granulosa cells were cultured in the absence (con) and presence of TGFα (α; 10 ng/ml) and TGFβ (β; 20 ng/ml) for 8 h. Total RNA (10μg/lane) were hybridized with cDNA probes for COX I and COX II. Densitometric analysis of COX II and 28S rRNA was performed using a Molecular Dynamics Phosphoimager. Data were normalized with the respective 28S and expressed as the percentage of control. Upper panel shows a representative filter and lower panel shows the mean±SEM of three experiments. *P<0.05, **P<0.01 (compared to control); ++P<0.01 (compared to TGFα stimulation)


FL
ES-6
con
α
β
α+β
con
α
β
α+β
COX II
0.1 ng
1 ng
10ng
COX I
28S
18S

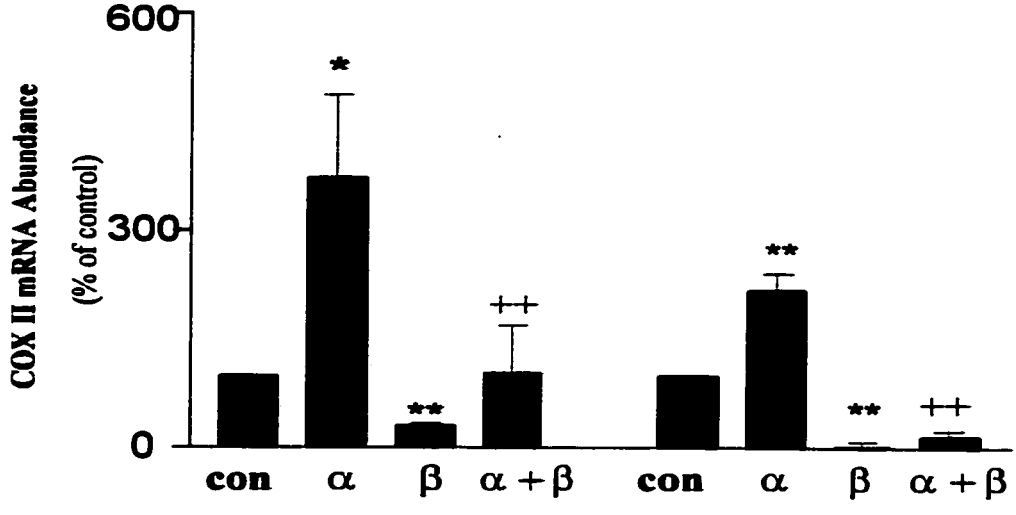
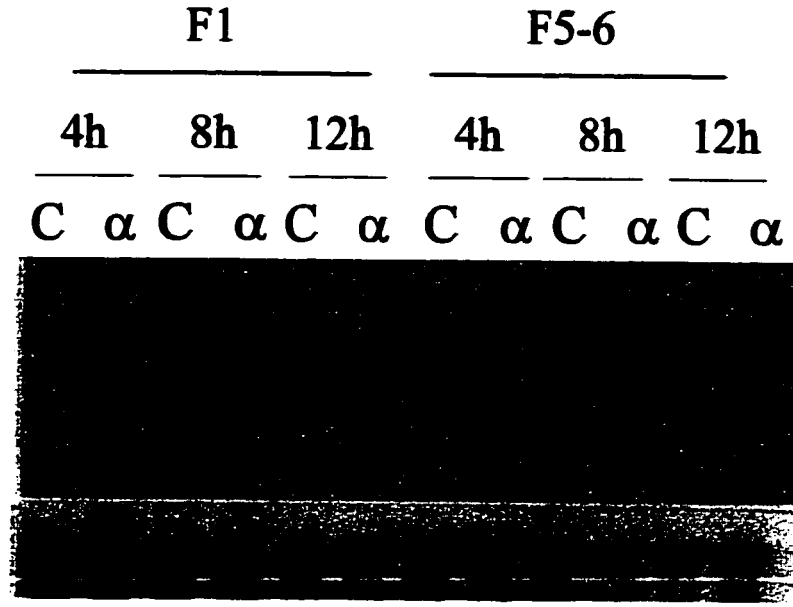
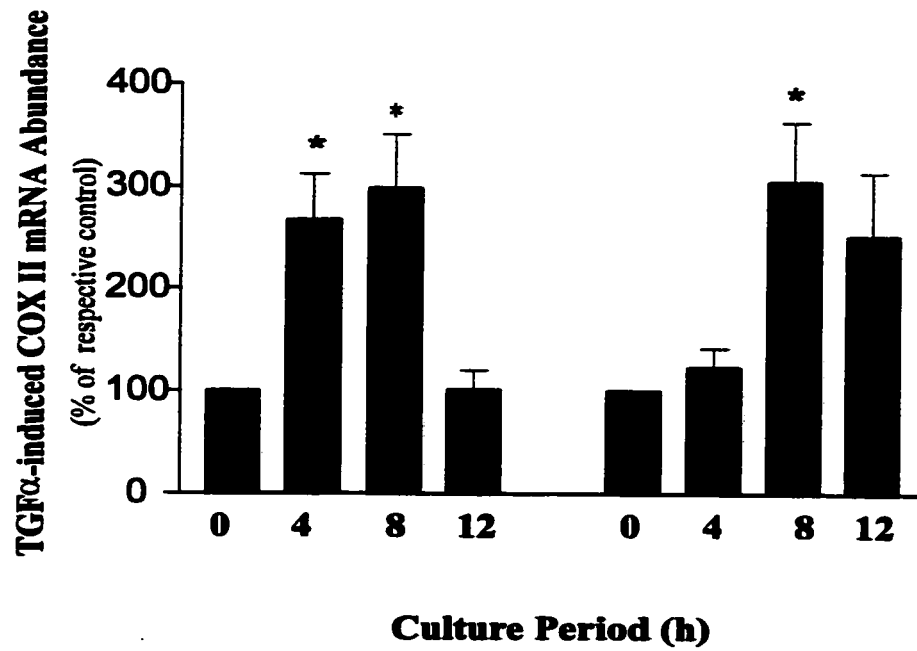

COX II mRNA Abundance
(% of control)
600
300
0
*
**
++
**
**
++
con
α
β
α+β
con
α
β
α+β

Figure 26: Time course study of the effect of TGFα (10 ng/ml) on granulosa cell COX II mRNA abundance. Granulosa cells from F_1 follicles were cultured for up to 12 h in the absence (c) and presence (α) of TGFα. Total RNA (8 μg/lane) were hybridized with a cDNA probe for COX II. Densitometric analysis of COX II and 28S rRNA was performed using a Molecular Dynamics Phosphoimager. Data were normalized with the respective 28S and expressed as the percentage of respective control. Upper panel shows a representative filter and lower panel shows the mean±SEM of three experiments. *P<0.05 (compared to respective control)


F1
F5-6
4h
8h
12h
4h
8h
12h
C α C α C α C α C α C α
COX II
28s


TGFα-induced COX II mRNA Abundance
(% of respective control)
400
300
200
100
0
*
*
*
0
4
8
12
0
4
8
12
Culture Period (h)

granulosa cells from F1 and F5-6 in the absence of the growth factors was variable and appeared to be independent of follicular maturation (data not shown).

In contrast, COX I mRNA was not detectable irrespective of the presence of the growth factors, the developmental stage of the follicle (Fig. 25) and the duration of culture (data not shown). This was probably due to a low abundance of the COX I message in this cell type, since as low as 0.1 ng of a fragment of this hen COX I cDNA was readily recognized in a dot blot assay, using the same probe (Fig. 25).

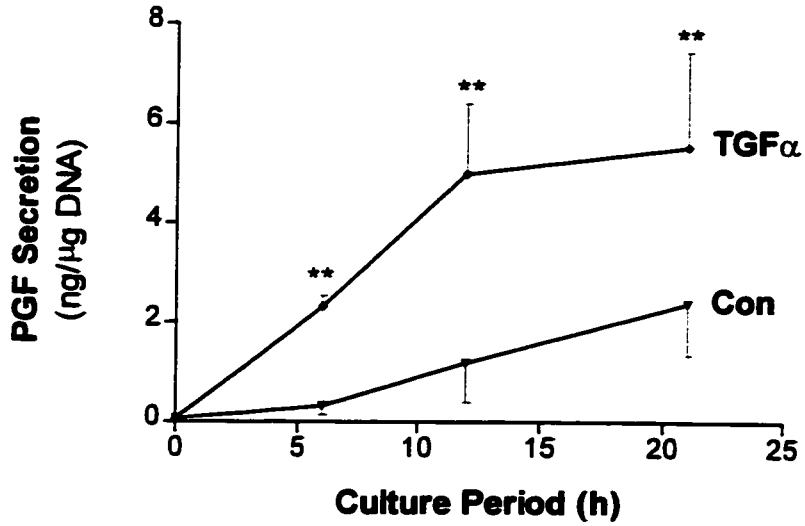
2. *Influence of TGFα and TGFβ on COX II protein content and PG synthesis*

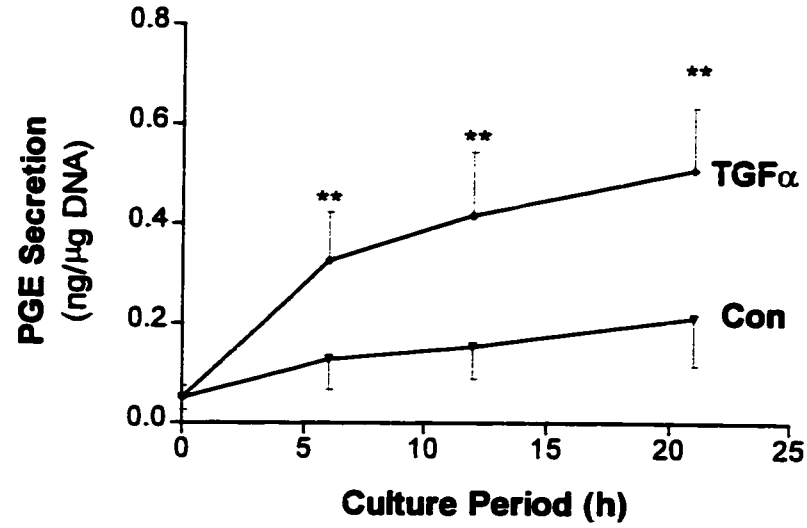
To determine if the changes in granulosa cell COX II mRNA abundance in the presence of the growth factors are accompanied by alterations in subsequent down-stream events, COX II protein content and PG production in granulosa cell cultures were assessed. Western blot analysis with a polyclonal anti-chicken COX II antibody indicates the presence of low levels of an 84 KDa COX II protein and occasionally of a 53 KDa breakdown product in F1 granulosa cells cultured for up to 24 h in the absence of exogenous growth factors (Fig. 28). TGFα (10 ng/ml) transiently increased COX II protein (84 KDa) content in a time-dependent manner, with a maximal response observed at 6-12 h treatment (Fig. 27, lower panel). This response was temporally associated with increases in PG secretion, which reached maximal level by 6 h, remained relatively constant throughout the culture period (Fig. 27, upper & middle panels).

Figure 28 shows the influence of various concentrations of TGFα (0-10 ng/ml) on F1 granulosa cell COX II protein content and PG production *in vitro*. TGFα significantly elevated COX II protein level and the synthesis of PG in a concentration-dependent manner.

Interestingly, the increase in COX II protein content evident in the presence of TGFα (≥ 1 ng/ml) was also accompanied by the significant increase in both PGE and PGF accumulation ($P<0.01$; Fig. 28). Although PG secretion was stimulated by TGFα and inhibited by TGFβ in both stages of follicular development (Fig. 29, upper and middle panel), COX II content was increased by TGFα and this response was suppressed by TGFβ only in F1 granulosa cells (Fig. 29, bottom panel). These findings suggests that the regulation of F5-6 granulosa cell PG production may be more important at other level (ie. $cPLA_2$) of the PG production pathway.

Figure 27: Temporal relationship between COX II protein expression and PG secretion following TGFα stimulation. Granulosa cells from F_1 follicles were cultured in the absence (con) and presence of TGFα (10 ng/ml) for up to 24h. PGE and PGF in the culture medium were assayed by RIA and solubilized cell protein (100 μg/lane) was analyzed by Western blot, using an anti-hen COX II antibody. The upper and middle panels show the response in PG production and the lower panel shows the stimulation of COX II by TGFα at different durations of incubation. Densitometric analysis of COX II protein data was performed using a Molecular Dynamic Phosphoimager. Data represent mean±SEM of three (PG secretion) or four (COX II protein content) experiments. **$p<0.01$ (compared to control at respective time points)


PGF Secretion
(ng/μg DNA)
8
6
4
2
0
0
5
10
15
20
25
Culture Period (h)
**
**
**
TGFα
Con


PGE Secretion
(ng/μg DNA)
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
25
Culture Period (h)
**
**
**
TGFα
Con

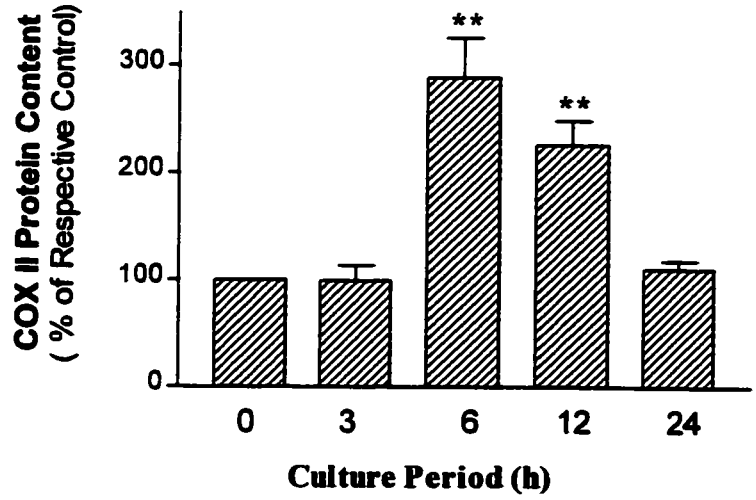

COX II Protein Content
(% of Respective Control)
300
200
100
0
0
3
6
12
24
Culture Period (h)
**
**

Figure 28: Dose-dependent increase in granulosa cell COX II protein content and PG secretion by TGFα. F_1 granulosa cells were cultured with different concentrations of TGFα (0-l0 ng/ml) for 21h (PG experiment), or 6h (COX II experiment). PGE and PGF in the culture media were assayed by RIA and solubilized cell proteins (100 μg/lane) were analyzed by Western blot. The lower panel shows the concentration-dependent response of PG production to TGFα. Values represent mean ±SEM of five experiments. *$p<0.05$, ** $p<0.01$ (compared to control). The middle panel indicates changes in COX II protein content as analyzed densitometrically, using the Molecular Dynamic Phosphoimager. Data represent mean±SEM of three experiments. *$p<0.05$ (compared to control). The upper panel shows a representitive blot of a concentration-response study on COX II (84 KDa) protein content following TGFα stimulation.

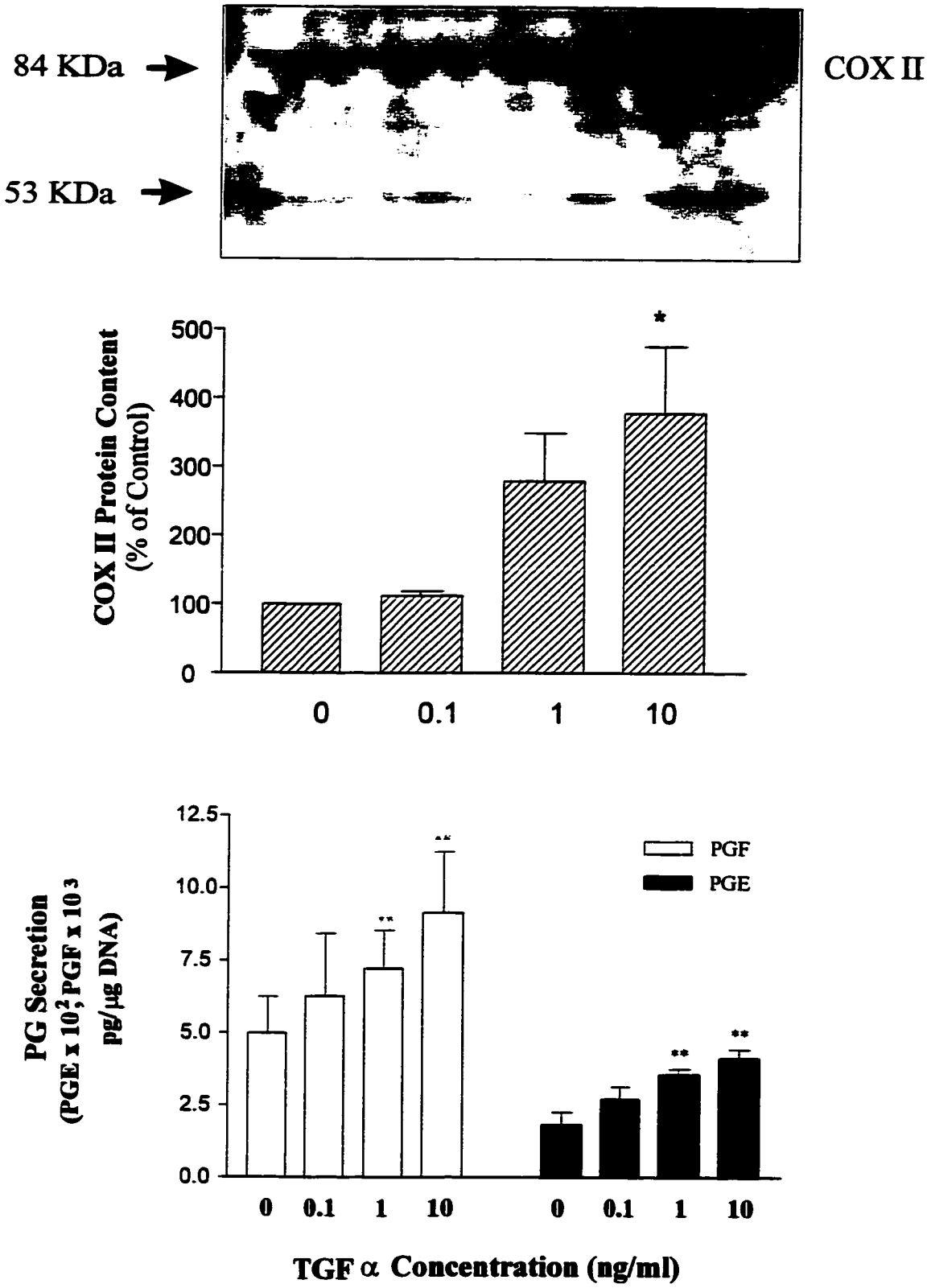
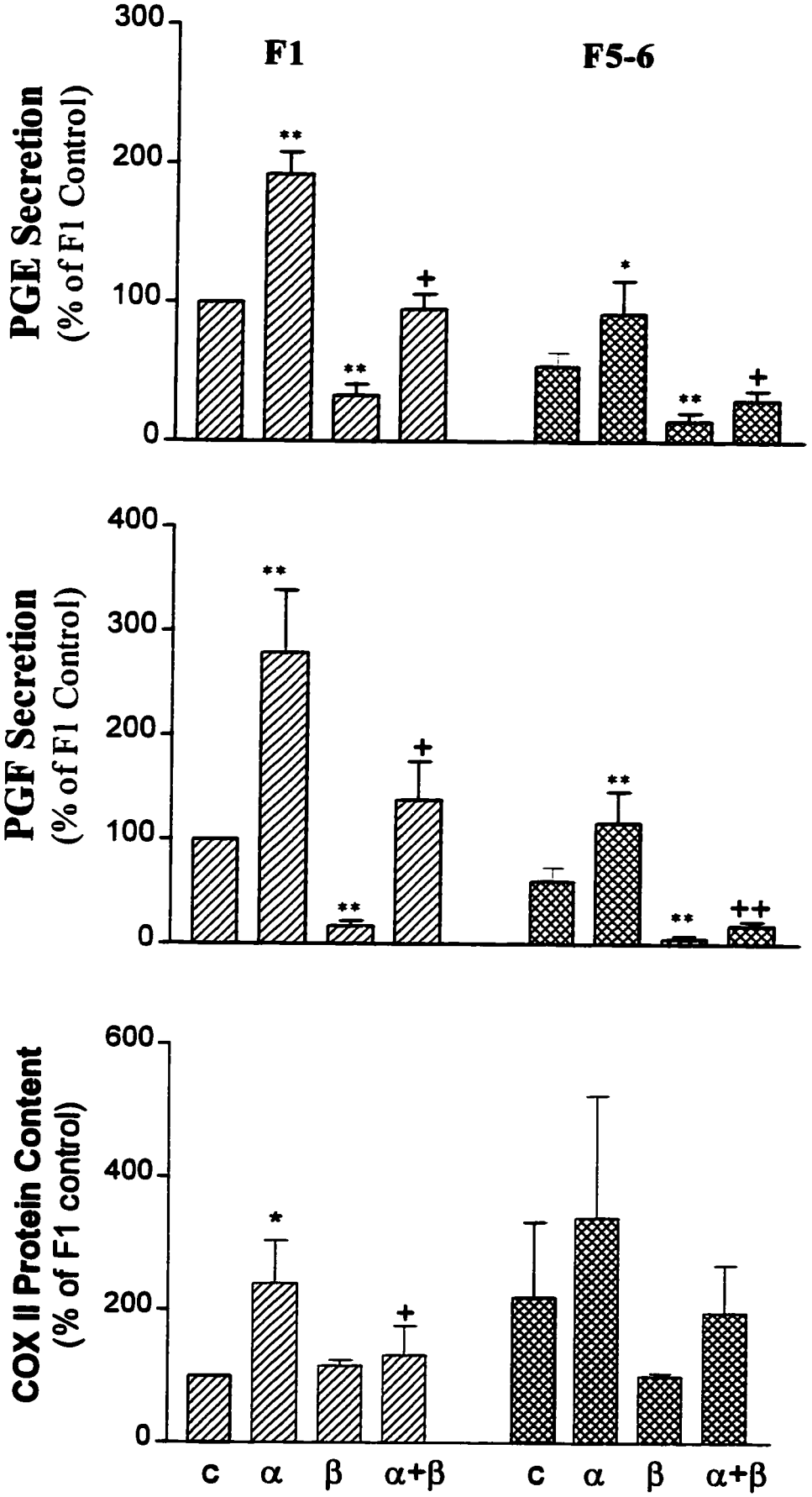

84 KDa
53 KDa
COX II
COX II Protein Content
(% of Control)
500
400
300
200
100
0
*
0
0.1
1
10
PG Secretion
(PGE x 10², PGF x 10³
pg/μg DNA)
12.5
10.0
7.5
5.0
2.5
0.0
PGF
PGE
**
**
**
**
0
0.1
1
10
0
0.1
1
10
TGF α Concentration (ng/ml)

Figure 29: Influence of TGFα and TGFβ on granulosa cell PG production and COX II protein expression during follicular development. Granulosa cells from F_1 and F5-6 follicles were cultured in the absence (C) or presence of TGFα(α) and/or TGFβ (β) for 21 h (PG experiment) or 6 h (COX II experiment). PGE and PGF in the culture media were assayed by RIA and solubilized cell proteins (100 μg/lane) were analyzed by Western blot. Data in the upper and middle panels show PG production in the absence and presence of the growth factors and represent mean ±SEM of six experiments; *$p<0.05$, **$p<0.01$ (compared to control), +$p<0.05$, ++$p<0.01$ (compared to TGFα stimulation). Densitometric analysis of COX II protein data, as illustrated in the lower panel, was performed using a Molecular Dynamic Phosphoimager. Data represent mean±SEM of five experiments. *$p<0.05$ (compared to control), +$p<0.05$ (compared to TGFα stimulation)


F1
F5-6
PGE Secretion
(% of F1 Control)
300
200
100
0
PGF Secretion
(% of F1 Control)
400
300
200
100
0
COX II Protein Content
(% of F1 control)
600
400
200
0
c
α
β
α+β
c
α
β
α+β

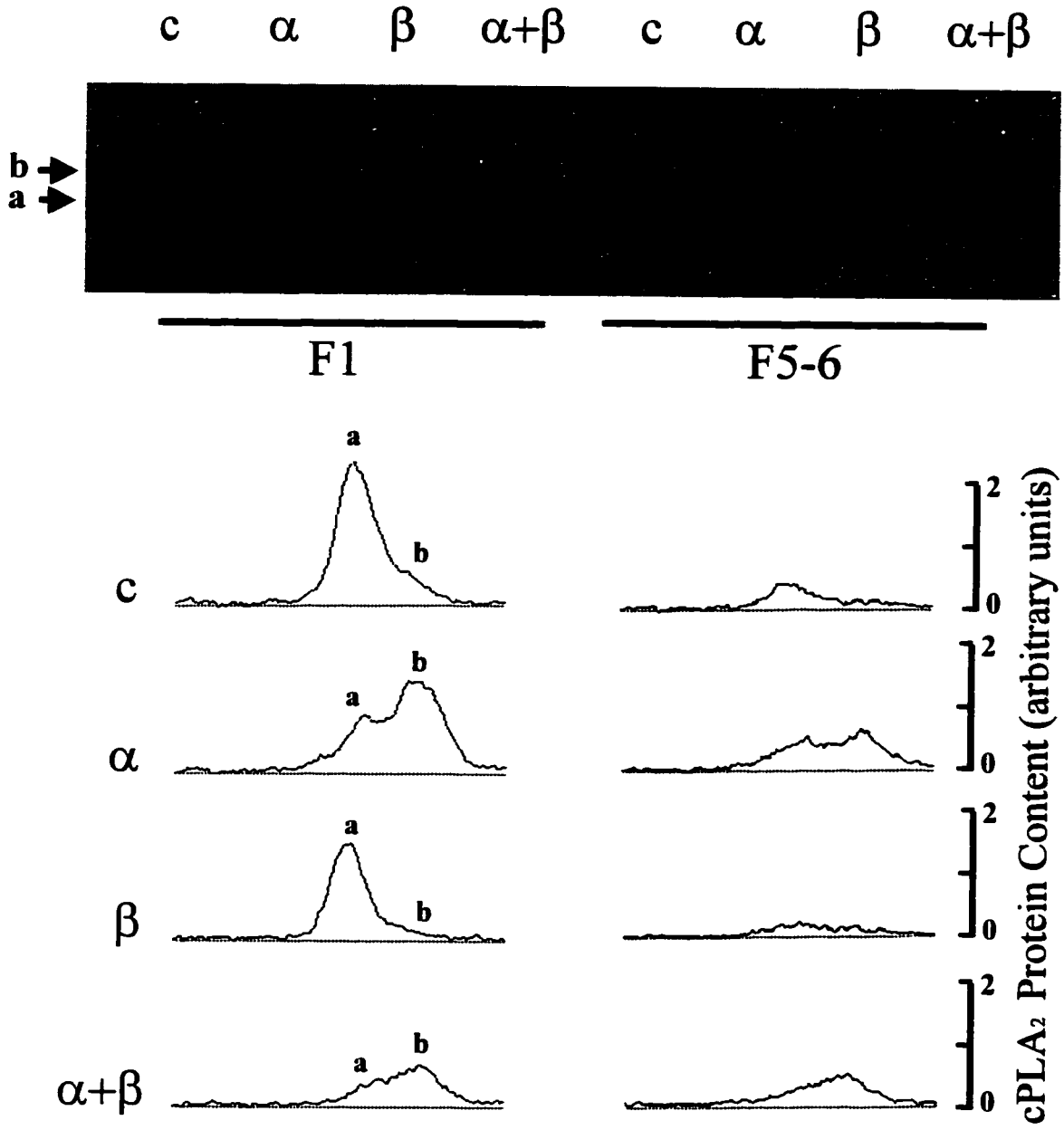
D. The regulation of PG production by TGFα and TGFβ is also at the level of $cPLA_2$

1. TGFα induces granulosa cell $cPLA_2$ phosphorylation during follicular development

To study the presence and the regulation of $cPLA_2$ in granulosa cells by TGFα and TGFβ *in vitro*, granulosa cells from F1 and F5-6 follicles, representing the more differentiated and proliferatively active populations of granulosa cells respectively, were cultured for 6 h in the absence and presence of TGFα and/or TGFβ. In the absence of exogenous transforming growth factors, granulosa cell $cPLA_2$ appeared as a 100 KDa protein doublet on a Western blot, with the most intense immuno-staining at the faster migrating band and the density of both bands increasing with follicular development (Fig. 30). Treatment with TGFα (10 ng/ml) caused a shift of $cPLA_2$ from a fast migrating form to a slow migrating one at both stages of follicular development, although both the faster migrating form and total $cPLA_2$ content were greater in granulosa cells from the more mature follicles (F1; Fig. 30). TGFβ caused a marked decrease in $cPLA_2$ protein content, but failed to prevent the change in the migration pattern of the protein induced by TGFα. This inhibition was more evident in granulosa cells from F5-6 follicles (Fig. 30).

It has been documented that the phosphorylation of serine residues (mainly Ser-505) of $cPLA_2$ alters the electrophoretic mobility of the enzyme (Clark et al., 1995) and this phosphorylation is induced by MAP kinase (Lin et al., 1993). In addition, staurosporin, a serine/threonine kinase inhibitor, blocks phosphorylation of $cPLA_2$ in intact cells and leads to decreased $cPLA_2$ activity (Bauldry et al., 1996). To verify whether the mobility shift observed with granulosa cell $cPLA_2$ in the presence of TGFα could possibly be associated with phosphorylation of the enzyme, granulosa cells were cultured for 30 min prior to and

Figure 30: Effect of TGFα and TGFβ on granulosa cell $cPLA_2$ protein content and electrophoretic mobility during follicular development. Granulosa cells from F1 and F5-6 were cultured for 6 h in the absence (c) and presence of TGFα (α; 10 ng/ml) and/or TGFβ (β; 20 ng/ml). Solubilized cell proteins (100 μg/lane) were analyzed by Western blot. *Upper panel:* A representative filter of Western blot analysis of $cPLA_2$ protein content. Arrows indicate a protein doublet of fast (a) and slow (b) migration forms. *Lower panel:* Densitometric scan of the Western blot. Peaks "a" and "b" correspond to the respective bands indicated in the upper panel



cPLA$_2$ Migration Pattern

during TGFα stimulation with 5 μM staurosporin, a concentration similar to one previously shown to inhibit $cPLA_2$ activity in human neutrophils (Bauldry et al., 1996) and not toxic to granulosa cells (as monitored by the trypan blue cell viability test). The TGFα-induced $cPLA_2$ mobility shift was effectively prevented by straurosporin, although its influence on total $cPLA_2$ protein content (high plus low mobility components) was variable (Fig. 31 A). Moreover, treatment of the granulosa cells with apigenin and PD98059, inhibitors of MAP kinase and MAP kinase kinase (MEK) respectively (Kuo and Yang, 1995; Morrison et al., 1996), likewise prevented the mobility shift of the enzyme (Fig. 31B). These findings suggest that the mobility shift of $cPLA_2$ induced by TGFα is associated with the phosphorylation of the protein, possibly via the action of MAP kinase.

2. *Regulation of granulosa cell $cPLA_2$ expression by TGFα and TGFβ during follicular development*

To investigate whether the interaction of TGFα and TGFβ also occurs at the transcriptional levels, $cPLA_2$ mRNA abundance was analyzed by Northern blot. Again, TGFβ significantly decreased $cPLA_2$ mRNA levels, with stronger inhibition observed in F5-6 granulosa cells. In contrast, TGFα failed to significantly ($p>0.05$) alter either basal or TGFβ suppression of $cPLA_2$ mRNA levels irrespective of follicular maturation (Fig. 32) and the duration of the culture period (data not shown).

The temporal influence of TGFβ on $cPLA_2$ expression during follicular development was also studied. Granulosa cells from F1 and F5-6 were cultured in the absence and presence of TGFβ for 0.5, 3 and 6 h and $cPLA_2$ mRNA abundance was analyzed by Northern blot. As depicted in Figure 33, TGFβ significantly decreased $cPLA_2$ transcript levels in a

time-dependent manner and significant inhibition was not observed until 6 h of culture (Fig. 33). Granulosa cell $cPLA_2$ protein expression was likewise suppressed by TGFβ in a time (Fig. 34)- and concentration (Fig. 35)- dependent fashion and was temporally associated with the decrease in mRNA level. By 24 h of culture, TGFβ suppressed $cPLA_2$ protein content to 37% and 20% of the controls in F1 and F5-6 granulosa cells, respectively (Fig. 34). Whereas 0.2 ng TGFβ/ml slightly (approximately 35%) but non-significantly decreased $cPLA_2$ protein content, over 80% of the enzyme protein was suppressed at a higher concentration (20 ng/ml; Fig. 35).

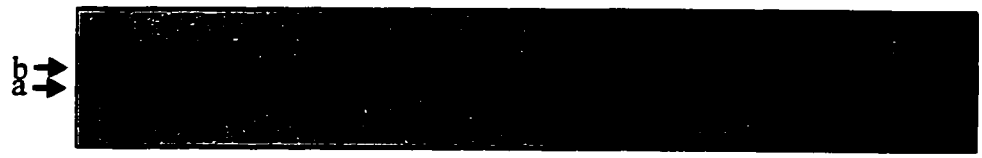
$cPLA_2$ hydrolyses sn-2 ester bonds of phospholipids, thereby releasing arachidonic acid for PG production. Coupled to the responses of $cPLA_2$ to TGFα and TGFβ, PGE production was likewise stimulated by TGFα and suppressed by TGFβ. Moreover, consistent with its influence on $cPLA_2$ and in contrast to the action of TGFα, the inhibition of PGF production by TGFβ was more pronounced at the early stage of follicular development (Fig. 7).

Figure 31: Western blot analysis of the effects of inhibitors of serine/threonine kinase and MAP kinase pathway on the changes in $cPLA_2$ migration pattern induced by TGFα. Inhibitors of serine/threonine kinase (Panel A) and MAP kinase pathway (Panel B; representative filter from 3 experiments): granulosa cells from F1 follicles were cultured for 6 h in the absence and presence of TGFα (10ng/ml) and/or staurosporin (5μM; Panel A) or apigenin (100 μM; Panel B) or PD98059 (20 μM; Panel B) [added 30 min before and during TGFα treatment] . Arrows in Panel B indicate protein doublet of fast (a) and slow (b) migrating forms. Peaks "a" and "b" in Panel A correspond to the fast and slow migrating $cPLA_2$ forms, respectively.

A

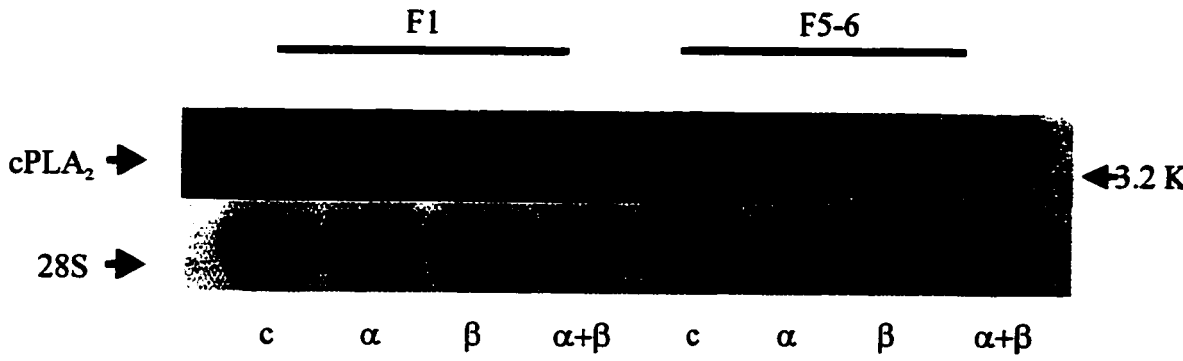
Con TGFα TGFα + Staurosporin

B



Con TGFα TGFα + Apigenin TGFα + PD 98059

Figure 32: Influence of transforming growth factors on $cPLA_2$ transcript levels in granulosa cells during follicular development. Granulosa cells from F1 and F5-6 follicles were cultured for 6 h in the absence (c) and presence of TGFα (α; 10 ng/ml) and/or TGFβ (β; 20 ng/ml). Total RNA (8 μg/lane) were hybridized with a cDNA probe for $cPLA_2$. Densitometric analysis of $cPLA_2$ and 28S rRNA was performed, using the Image Analysis Systems from Bio-Rad Laboratories. Data were normalized with the respective 28S and expressed as the percentage of F1 control. *Upper panel* shows a representative filter and *lower panel* shows the mean±SEM of four experiments. *$p<0.05$ (compared to control)


F1
F5-6
cPLA2
28S
3.2 K
c
α
β
α+β
c
α
β
α+β

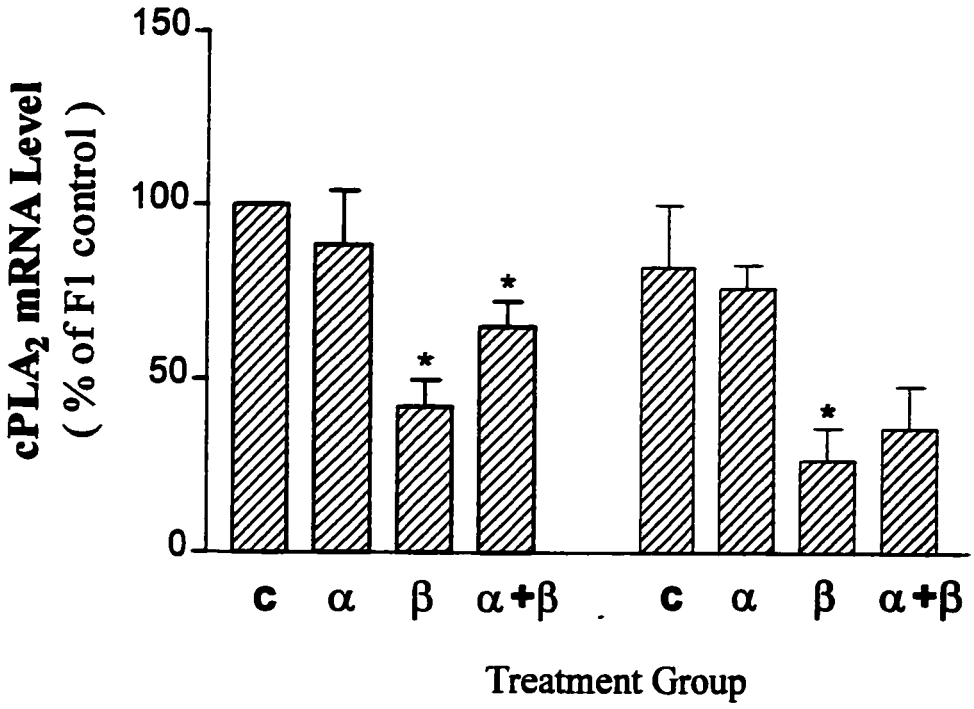

cPLA2 mRNA Level
(% of F1 control)
150
100
50
0
*
*
*
c
α
β
α+β
c
α
β
α+β
Treatment Group

Figure 33: Time course of the effect of TGFβ on $cPLA_2$ mRNA abundance in granulosa cells during follicular development. Granulosa cells from F1 and F5-6 follicles were cultured for up to 6 h in the absence (c) and presence of TGFβ (β; 20 ng/ml). Total RNA (8 μg/lane) was hybridized with a cDNA probe for $cPLA_2$. Densitometric analysis of $cPLA_2$ and 28S rRNA was performed using the Image Analysis Systems from Bio-Rad Laboratories. Data were normalized with the respective 28S and expressed as the percentage of F1 control. *Upper panel* shows a representative filter and *lower panel* shows the mean±SEM of five experiments. *$p<0.05$ (compared to control)

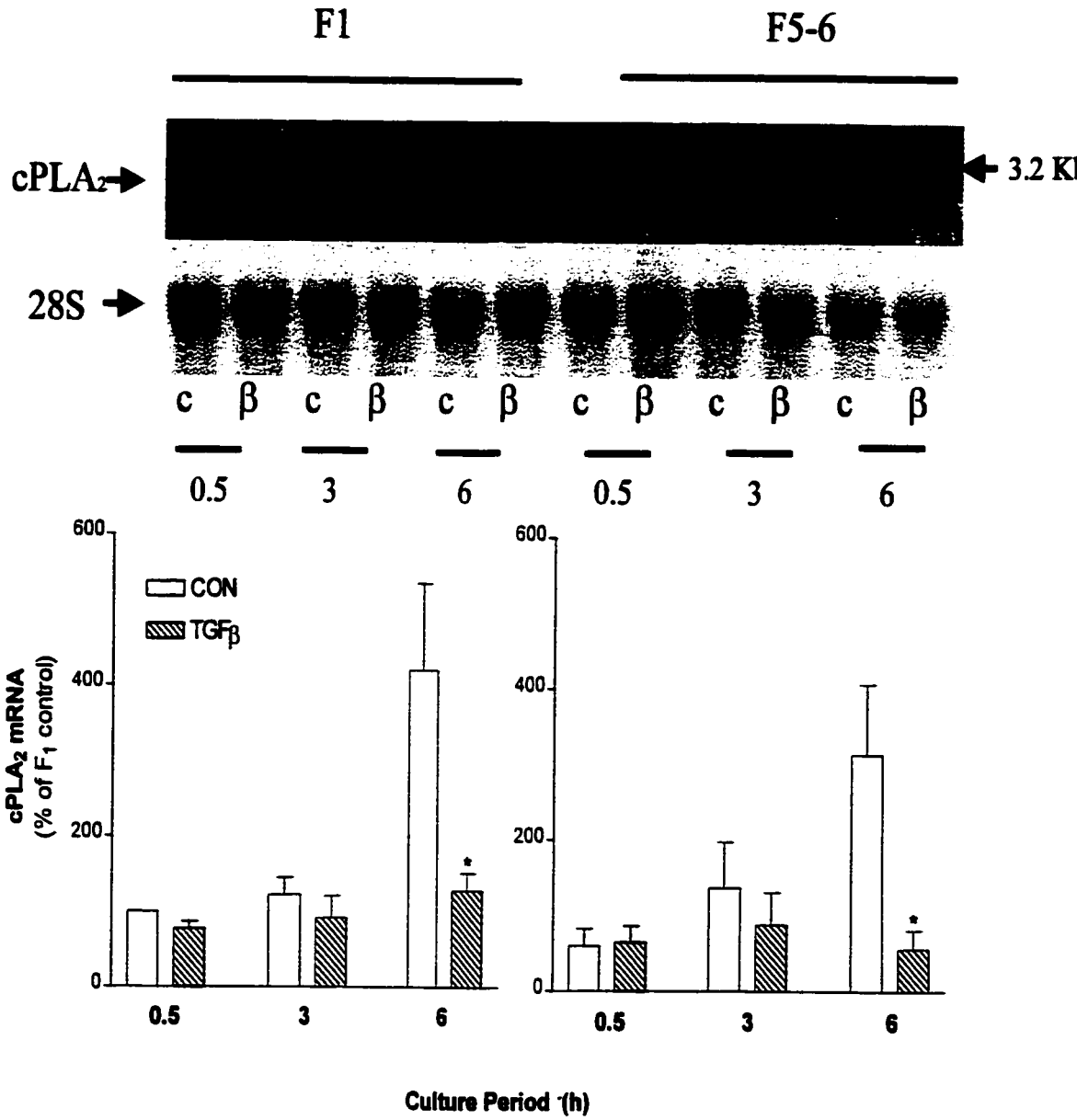
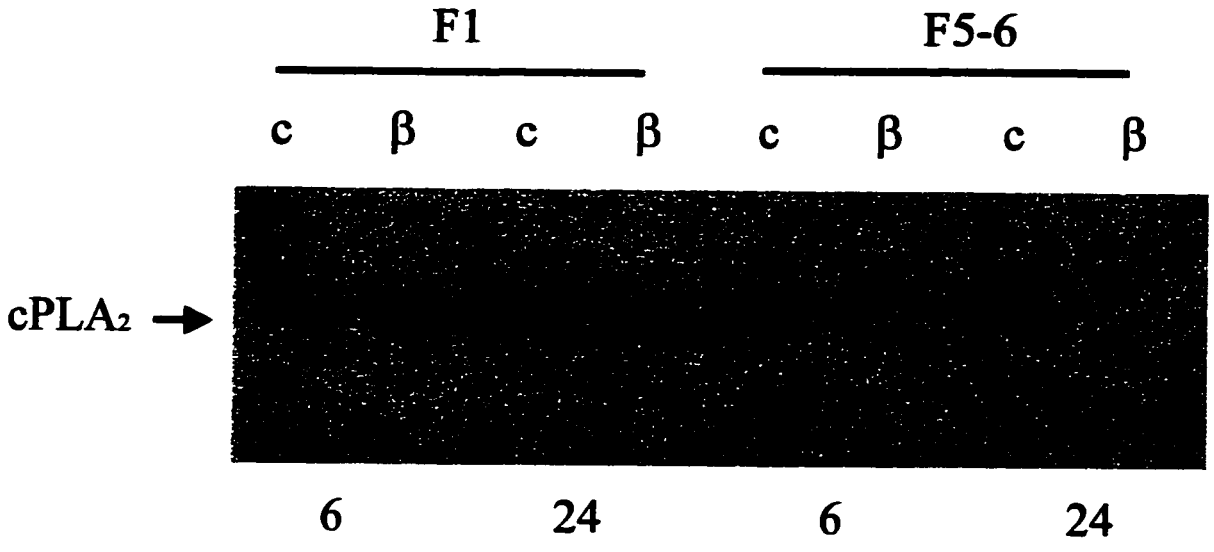

F1
F5-6
cPLA2
3.2 K
28S
c β c β c β c β c β c β
0.5 3 6 0.5 3 6
600
400
200
0
CON
TGFβ
cPLA2 mRNA
(% of F1 control)
*
*
0.5 3 6
0.5 3 6
Culture Period (h)

Figure 34: Temporal influence of TGFβ on $cPLA_2$ protein expression in granulosa cells during follicular development. Granulosa cells from F1 and F5-6 follicles were cultured for up to 24 h in the absence (c) and presence of TGFβ (β; 20 ng/ml). Equal amounts of cellular protein (60-100μg protein/lane, depending on the specific experiment) were analysed by Western blot. *Upper panel* shows a representative Western blot and *lower panel* illustrates changes in $cPLA_2$ protein content, as analysed densitometrically with the Image Analysis Systems from Bio-Rad Laboratories. Data represent mean±SEM of four experiments. *$p<0.05$ (compared to respective controls)


F1
F5-6
c
β
c
β
c
β
c
β
cPLA2
6
24
6
24

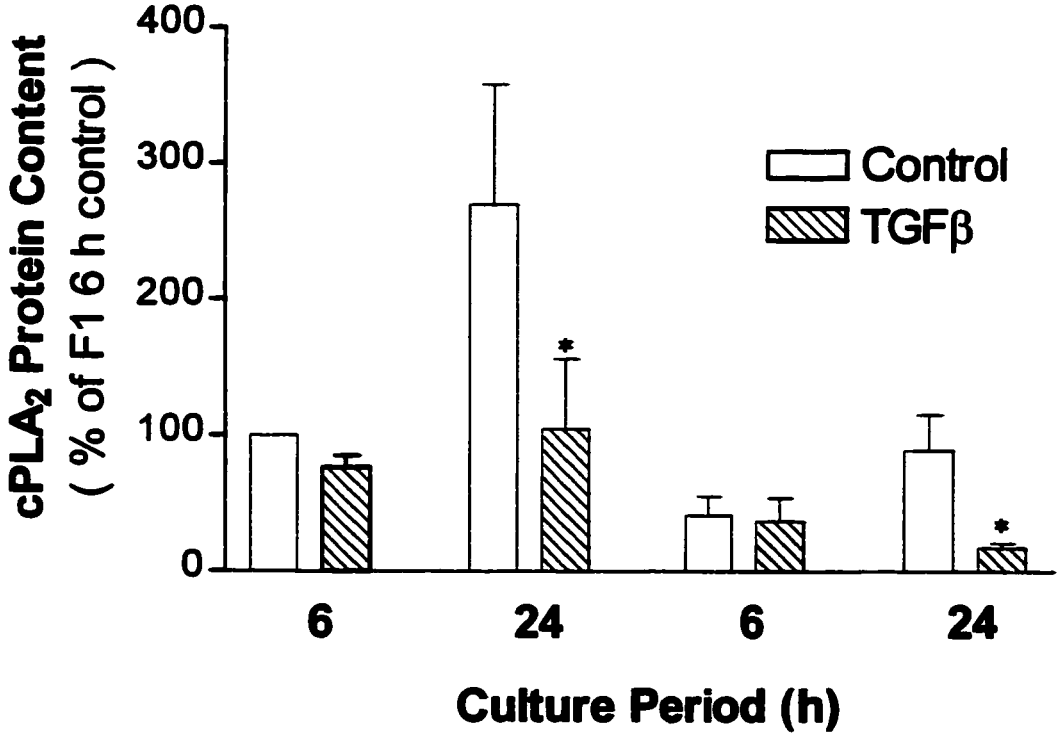
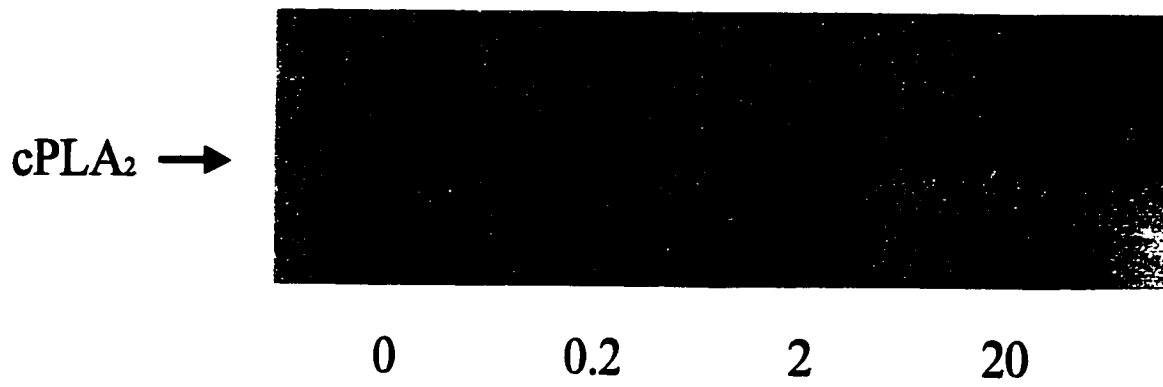
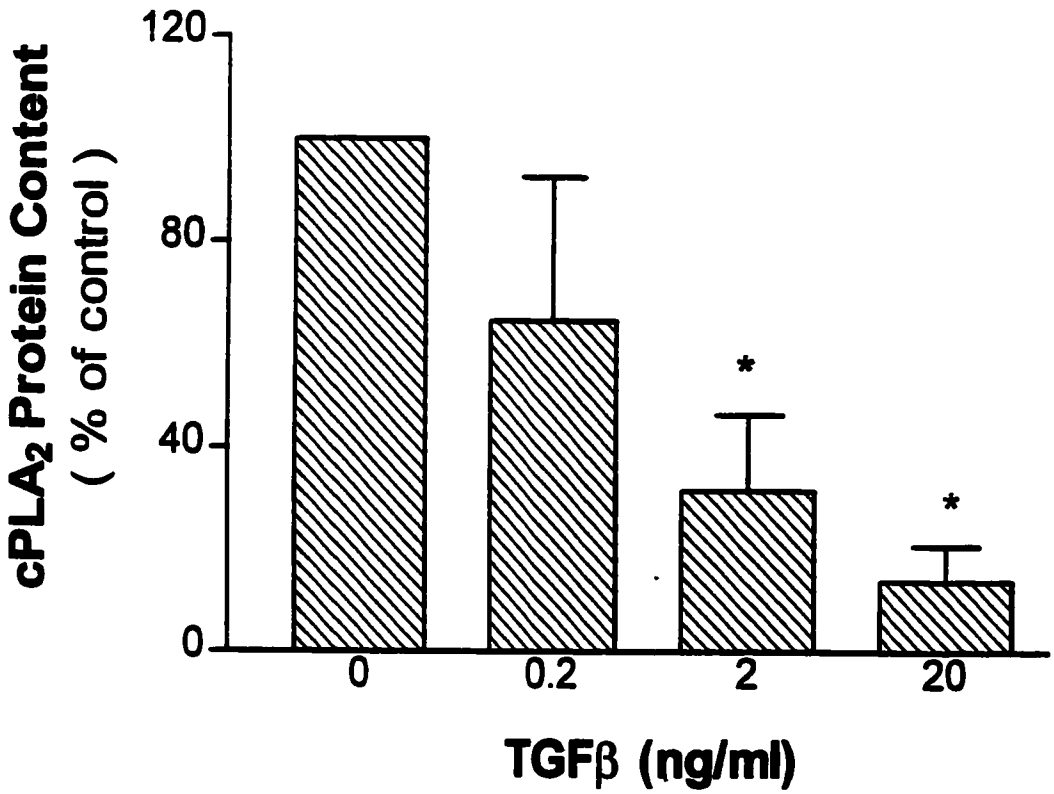

cPLA2 Protein Content
(% of F1 6 h control)
400
300
200
100
0
Control
TGFβ
*
*
6
24
6
24
Culture Period (h)

Figure 35: Concentration-dependent suppresion of granulosa cell $cPLA_2$ protein content by TGFβ *in vitro*. Granulosa cells from F5-6 follicles were cultured for 24 h in the presence of different concentration of TGFβ (0-20 ng/ml). Equal amounts of cellular protein (60-100μg protein/lane, depending on the specific experiment) were analysed by Western blot. *Upper panel:* Representative filter of a dose-response study. *Lower panel:* Changes in $cPLA_2$ protein content, as analysed densitometrically with the Image Analysis Systems from Bio-Rad Laboratories. Data represent mean±SEM of three experiments. *$p<0.05$ (compared to control)


cPLA2
0
0.2
2
20
TGFβ (ng/ml)


cPLA2 Protein Content
(% of control)
120
80
40
0
*
*
0
0.2
2
20
TGFβ (ng/ml)

E. Importance of autoregulation of Mother Against dpp Related protein 2 (MADR2) by TGFβ in conferring follicular-stage dependent granulosa cell $cPLA_2$ suppression by the growth factor

1. In vivo expression of MADR2 in granulosa cells at different stages of follicular development

To determine if MADR2 is expressed in granulosa cells *in vivo* and if its cellular content is dependent on the stage of follicular development, granulosa cells from F1, F3 and F5-6 follicles and LWF (follicles awaiting selection and recruitment into the developmental pool; undifferentiated cells) were isolated and proteins were extracted for Western analysis. As shown in Figure 36, MADR2 appeared as a 60 kDa protein in granulosa cells at all stages of follicular development examined. Expression of this protein was minimal in granulosa cells from LWF, highest in F5-6 granulosa cells, and decreased with further follicular development (Fig. 36).

2. Regulation of MADR2 expression by TGFβ

MADR2 is a component of the activin and TGFβ signalling systems (Eppert et al 1996; Baker & Harland 1996) and functions as a transcriptional co-activator in target cell gene transcription (Liu et al 1996; Chen et al., 1996b). Thus, we examined whether MADR2 expression in granulosa cells was regulated by TGFβ *in vitro* at both transcriptional and translational levels. MADR2 protein content in F1 granulosa cells was significantly increased by TGFβ in a concentration-dependent manner ($P<0.05$), reaching a level 400 % above the control at 20 ng/ml ($p < 0.05$; Fig. 37), while the abundance of MADR2 transcript (~3.6 kb) was increased 7.5 fold by the growth factor ($p < 0.05$; Fig. 38).

Figure 36: MADR2 protein content in granulosa cells at different stages of follicular development *in vivo*. Granulosa cells were isolated from F1, F3, F5-6 and LWF follicles and equal amounts of cellular protein (60-100μg protein/lane, depending on the specific experiment) were analysed by Western blot. *Upper panel:* Representative filter of MADR2 protein. *Lower panel:* Changes in MADR2 protein content as analysed densitometrically, using the Image Analysis Systems from Bio-Rad Laboratories. Data are expressed as the percentage of F1 (100%) and represent mean±SEM of four experiments. Different letter subscripts indicate statistically significant differences ($p < 0.05$).


60 kDa
MAD

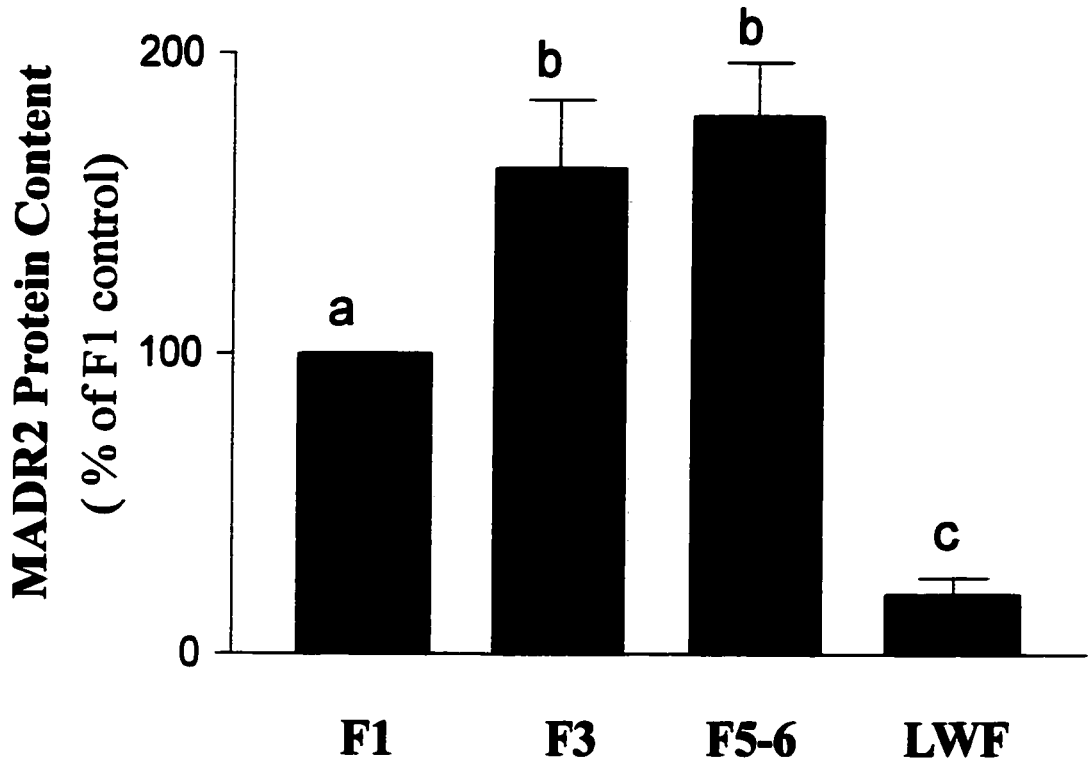
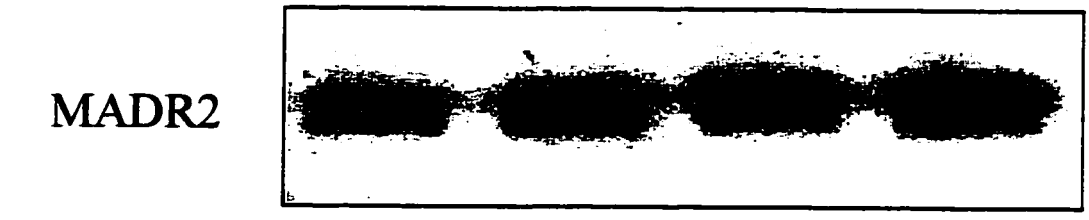

MADR2 Protein Content
(% of F1 control)
200
100
0
a
b
b
c
F1
F3
F5-6
LWF

Figure 37: Concentration-dependent study on the increase in granulosa cell MADR2 protein content induced by TGFβ *in vitro*. Granulosa cells from F1 follicles were cultured for 12 h with various concentrations of TGFβ (0-20 ng/ml). Equal amounts of cellular protein (60-100μg protein/lane, depending on the specific experiment) were analysed by Western blot. *Upper panel:* Representative filter of the concentration-response study. *Lower panel:* Changes in MADR2 protein content as analysed densitometrically, using the Image Analysis Systems from Bio-Rad Laboratories. Data are expressed as the percentage of control (100%) and represent mean±SEM of four experiments. ** $p < 0.01$ (compared to control)


MADR2

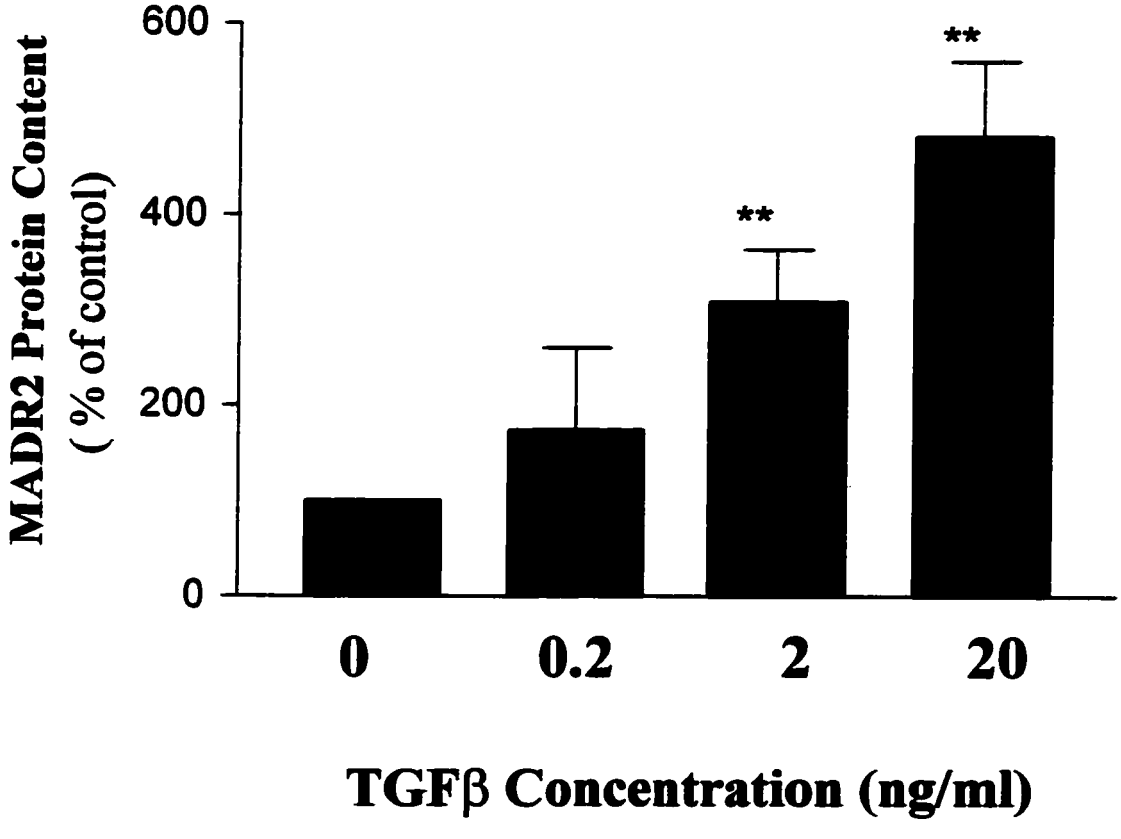
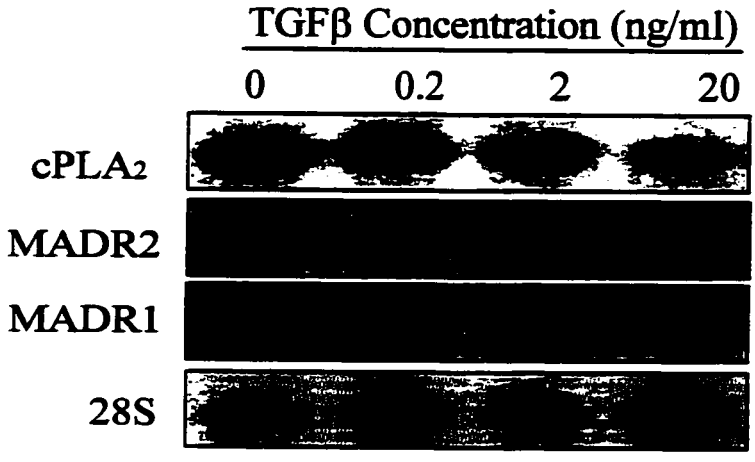
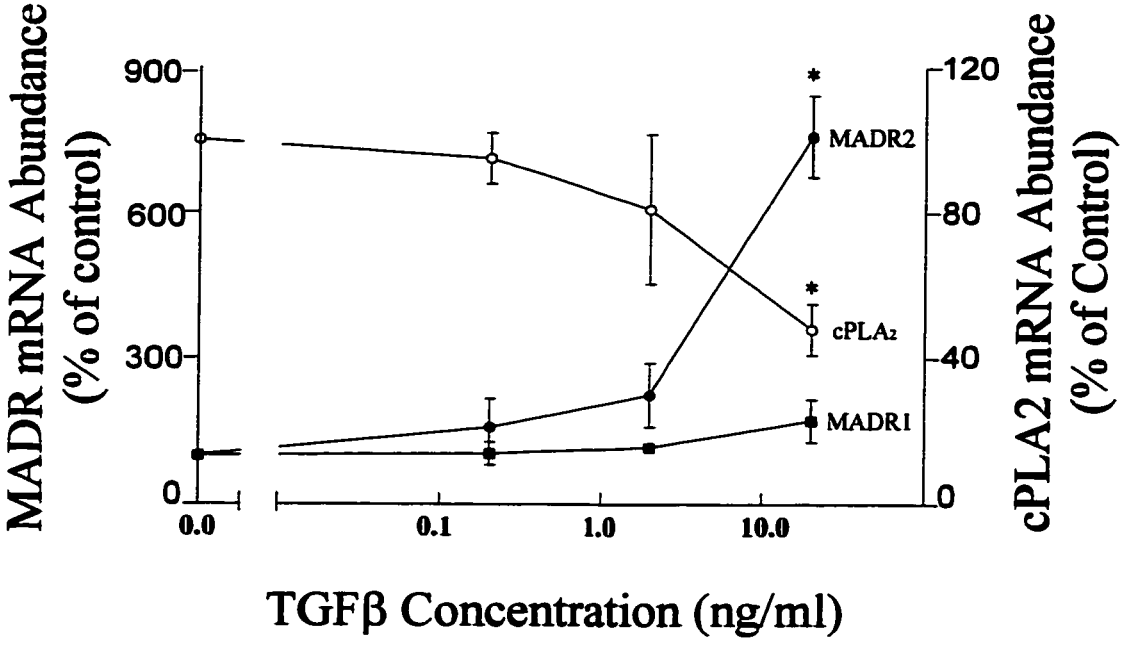

MADR2 Protein Content
(% of control)
600
400
200
0
**
**
0
0.2
2
20
TGFβ Concentration (ng/ml)

Figure 38: Concentration-dependent study on the influence of TGFβ on granulosa cell MADR2, MADR1 and $cPLA_2$ transcript levels *in vitro*. Granulosa cells from F1 were cultured for 6 h with TGFβ (0-20 ng/ml). Total RNA (10 μg/lane) was hybridized with the respective cDNA probes and densitometric analysis of MADR1, MADR2 and $cPLA_2$ mRNA and 28S rRNA was performed, using the Image Analysis Systems from Bio-Rad Laboratories. *Upper panel* shows representative filters and *lower panel* shows changes in MADR1, MADR2 and $cPLA_2$ mRNA abundance. Data are normalized with the respective 28S, expressed as the percentage of control (100%) and represent mean±SEM of four experiments. *$p<0.05$ (compared to control)


TGFβ Concentration (ng/ml)
0
0.2
2
20
cPLA2
MADR2
MADR1
28S


MADR mRNA Abundance
(% of control)
900
600
300
0
120
80
40
0
cPLA2 mRNA Abundance
(% of Control)
MADR2
cPLA2
MADR1
*
*
0.0
0.1
1.0
10.0
TGFβ Concentration (ng/ml)

MADR1(~55kDa), a MAD-related protein specifically phosphorylated by bone morphogenetic protein (BMP; Hoodless, et.al., 1996), was also present in granulosa cells. It has a transcript size of 3.6 kb but was not significantly affected by the presence of TGFβ at concentrations as high as 20 ng/ml ($p > 0.05$; Fig. 38). In addition, the presence of MADR1 and MADR2 in the hen granulosa cells appear authentic, as no detectable signals were evident in immunoblots when the anti-MADR1 and anti-MADR2 antibodies were replaced with their respective pre-immune sera (data not shown). Interestingly, the basal MADR2 transcript level was highest early in the culture period (0.5 h) but significantly decreased with the duration of culture (Fig. 39). The addition of TGFβ (20 ng/ml) to the cell cultures had no significant influence on granulosa cell MADR2 mRNA abundance in both F1 and F5-6 until 6 h of culture, when the transcript levels were significantly elevated by 250% (Fig. 39A) and 500% (Fig. 39B), respectively. Thus, granulosa cell MADR2 is selectively regulated by TGFβ at both transcriptional and translational levels.

3. Follicular stage dependence of MADR2 response to TGFβ

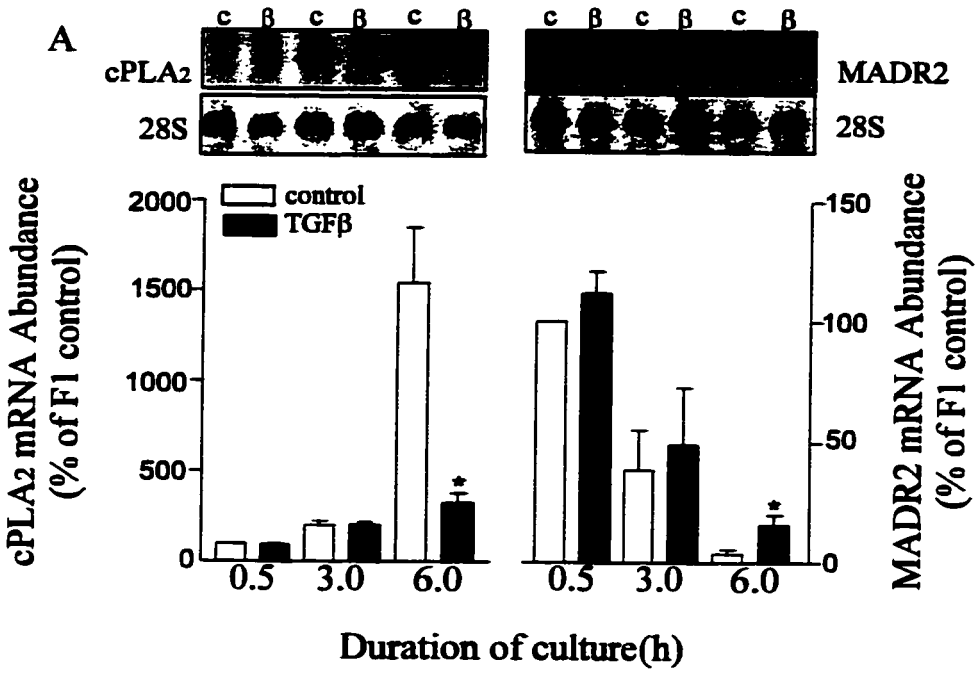
The regulation of granulosa cell MADR2 expression by TGFβ was found to be dependent on the stage of follicular development. While the growth factor significantly increased MADR2 mRNA abundance at 6 h (Fig. 39) and protein content at 12 h (Fig. 40) in granulosa cells in both F1 and F5-6 follicles ($p< 0.05$), MADR2 expression in the presence of TGFβ (20ng/ml) appeared to be higher in the less differentiated cells (F5-6; Fig. 39 & 40). This is supported by the outcome of statistical analysis by ANOVA, showing a significant interaction between follicular stage and TGFβ effect ($p< 0.05$), presumably brought about by the greater stimulatory action of the growth factor at the F5-6 stage (Fig. 39). Similarly,

MADR2 protein content in F5-6 granulosa cells in the presence of TGFβ (20ng/ml) was two times higher than in the F1 cells (Fig.40). In contrast, TGFβ had no significant influence on granulosa cell MADR1 protein content irrespective of the stage of follicular development ($p > 0.05$; Fig.40).

4. Reciprocal expression of MADR2 and $cPLA_2$ in granulosa cells cultured in the absence and presence of TGFβ

To investigate whether the changes in $cPLA_2$ expression in hen granulosa cells by TGFβ could possibly be associated with the up-regulation of MADR2, experiments were performed to compare their concentration-dependent and temporal responses to TGFβ. In the absence of TGFβ, $cPLA_2$ mRNA abundance increased while MADR2 mRNA levels decreased with the duration of culture, with the earliest significant changes observed at the same time point (6 h; Fig. 39). The addition of TGFβ to the cultures increased MADR2 mRNA abundance and decreased that of $cPLA_2$ following 6 h of culture when compared to their respective controls (Fig. 39). The divergent effects of TGFβ on the expression of MADR2 and $cPLA_2$ were both concentration-dependent, with significant responses evident at the same concentration of the growth factor (20ng/ml; Fig. 38). Thus, the decrease of $cPLA_2$ expression is coupled to the increase of MADR2 expression in granulosa cells during follicular development.

Figure 39: Time course studies on the effects of TGFβ on MADR2 and $cPLA_2$ mRNA abundance in granulosa cells *in vitro*. Granulosa cells from F1 (A) and F5-6 (B) were cultured for up to 6 h in the absence (C) and presence (β) of TGFβ (20 ng/ml). Total RNA (10 μg/lane) was hybridized with cDNA probe for $cPLA_2$ and MADR2 and densitometric analysis of MADR2 and $cPLA_2$ mRNA and 28S rRNA was performed, using the Image Analysis Systems from Bio-Rad Laboratories. *Upper panel:* Representative filters of Northern blots. *Lower panel:* Data are normalized with the respective 28S, expressed as the percentage of F1 control and shown as mean±SEM of five experiments. *$p<0.05$ (compared to control)


A
c β c β c β
c β c β c β
cPLA2
28S
MADR2
28S
control
TGFβ
2000
1500
1000
500
0
150
100
50
0
0.5 3.0 6.0
0.5 3.0 6.0
cPLA2 mRNA Abundance (% of F1 control)
MADR2 mRNA Abundance (% of F1 control)
Duration of culture(h)

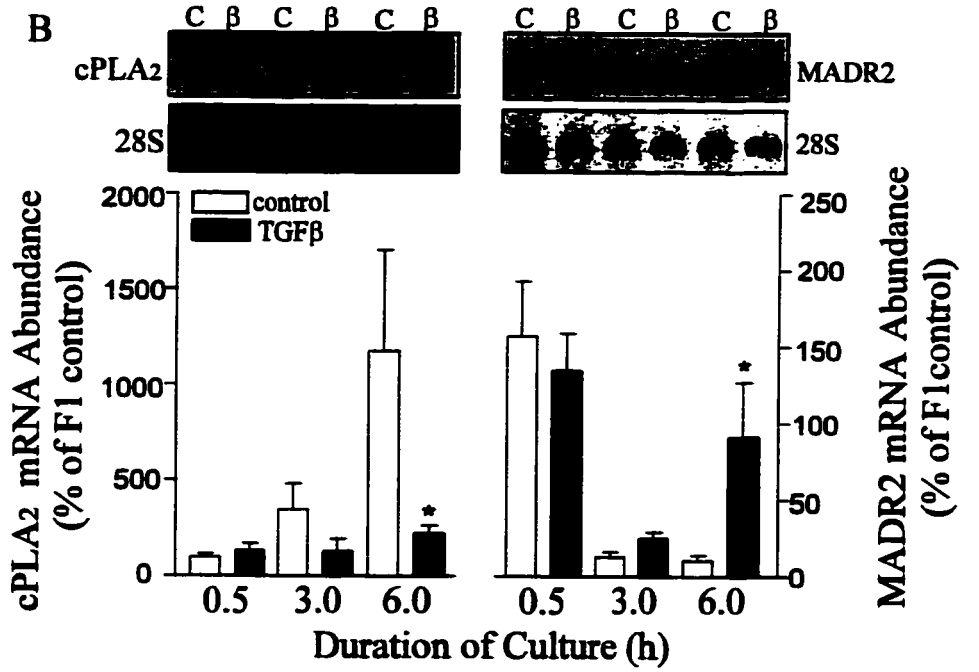
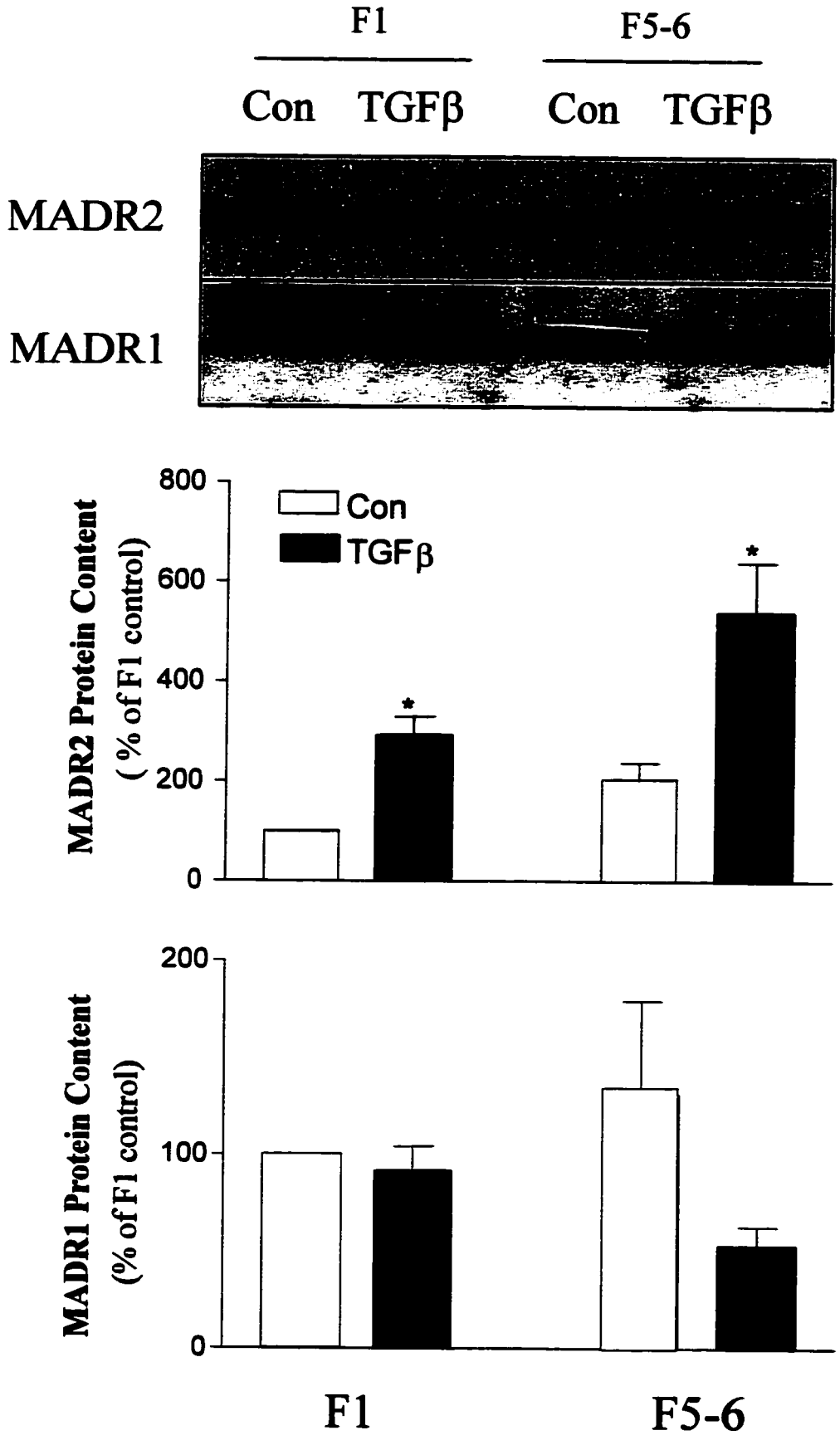

B
C β C β C β
C β C β C β
cPLA2
28S
MADR2
28S
control
TGFβ
2000
1500
1000
500
0
250
200
150
100
50
0
0.5 3.0 6.0
0.5 3.0 6.0
cPLA2 mRNA Abundance (% of F1 control)
MADR2 mRNA Abundance (% of F1control)
Duration of Culture (h)

Figure 40: Influence of TGFβ on granulosa cell MADR1 and MADR2 protein expression during follicular development. Granulosa cells from F1 and F5-6 follicles were cultured for 12 h in the absence (con) and presence of TGFβ (20 ng/ml). Equal amounts of cellular protein (60-100μg protein/lane, depending on the specific experiment) were analysed by Western blot. *Upper panel:* Representative Western blots. *Middle Panel:* Changes in MADR2 protein content. *Lower Panel:* Changes in MADR1 protein content. Densitometric analysis was carried out using the Image Analysis Systems from Bio-Rad Laboratories. Data are expressed as the percentage of respective F1 control (100%) and represent mean±SEM of four experiments; *$p<0.05$ (compared to respective control)


F1
F5-6
Con
TGFβ
Con
TGFβ
MADR2
MADR1
MADR2 Protein Content
(% of F1 control)
800
600
400
200
0
Con
TGFβ
*
*
MADR1 Protein Content
(% of F1 control)
200
100
0
F1
F5-6

F. Urokinase plasminogen activator (uPA) is up-regulated by TGFα

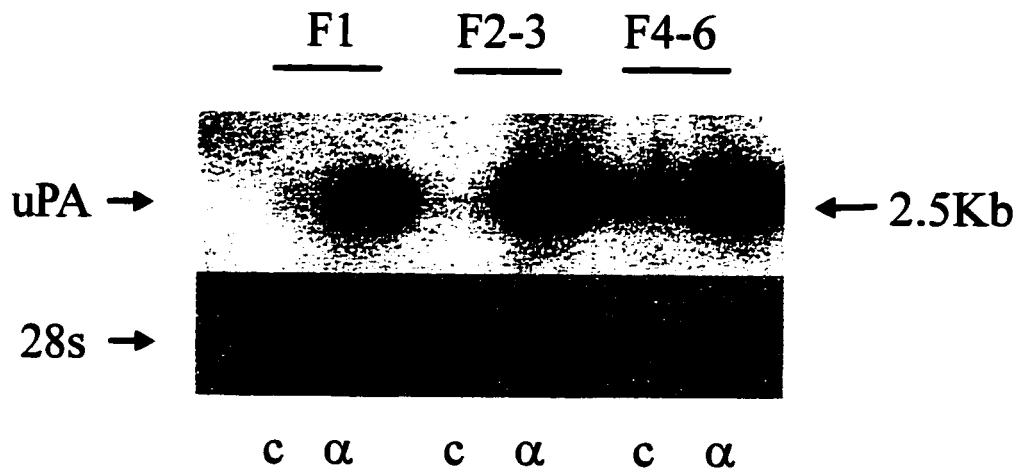
1. Follicular stage-dependent uPA mRNA expression and its regulation by TGFα

To determine the expression and response of granulosa cell uPA mRNA to TGFα during follicular development, granulosa cells from F1, F2-3 and F4-6 were cultured for 21 h in the absence and presence of TGFα , total RNA was then isolated and analyzed by Northern blot. Urokinase PA mRNA is present in granulosa cells from all stages of follicular development and is 2.5 kb in size (Fig. 41). The abundance of basal uPA mRNA was highest in granulosa cells from F4-6 but decreased with follicular maturation (ANOVA $p<0.05$). TGFα-stimulated uPA mRNA level was highest in F4-6 granulosa cells although the percentage increase was not as high as those from more mature follicles (Fig. 41; 35-, 42- and 3- fold in F1, F2-3 and F4-6, respectively).

2. Regulation of uPA protein content by TGFα

To determine if the changes in granulosa cell uPA mRNA abundance noted in the presence of TGFα are accompanied by alterations in protein level, uPA protein content was also examined. Western blot analysis with a polyclonal anti-chicken uPA antibody indicated the presence of a 35 kDa uPA protein in granulosa cells cultured for 21 h. Like uPA mRNA abundance, the basal uPA protein level was highest in granulosa cells from F4-6, and decreased with follicular development (ANOVA, $p<0.05$). In two out of three experiments, the uPA protein was not detectable in F1 granulosa cells. Similar to the uPA mRNA response, uPA protein level in the presence of the growth factor was also maximal in granulosa cells from early stages of follicular development (F4-6) although the percentage stimulation by TGFα was lower than that in more mature follicles (Fig. 42).

Figure 41: Effect of TGFα on uPA transcript levels in granulosa cells during follicular development. Granulosa cells from F1, F2-3 and F4-6 follicles were cultured for 21 h in the absence and presence of TGFα (10 ng/ml). Equal amounts of of total RNA (4-10 μg/lane, depending on the specific experiment) were hybridized with a chicken uPA cDNA probe. Densitometric analysis of uPA and 28S rRNA was performed using the Image Analysis Systems from Bio-Rad Laboratories. Data were normalized with the respective 28S and expressed as the percentage of F1 control. Basal F1 uPA mRNA expression was 5.49 ± 4.7 arbitrary units after normalization with 28S. *Upper panel* shows a representative filter and *lower panel* shows the mean±SEM of four experiments. "c" or "con", control; "α ", TGFα; *$p<0.05$, **$p<0.01$ (compared to respective controls)


F1
F2-3
F4-6
uPA
2.5Kb
28s
c α c α c α

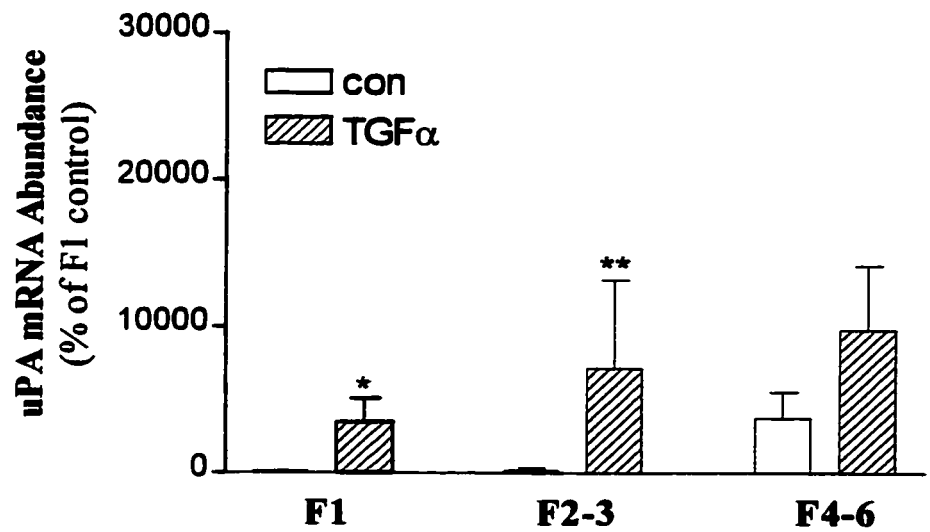
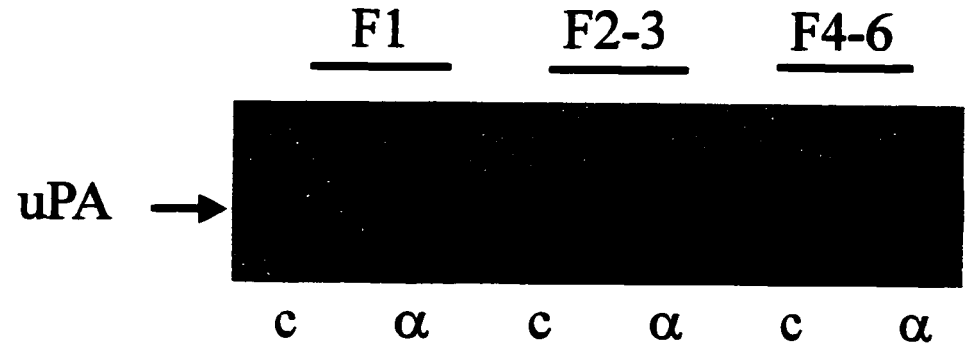
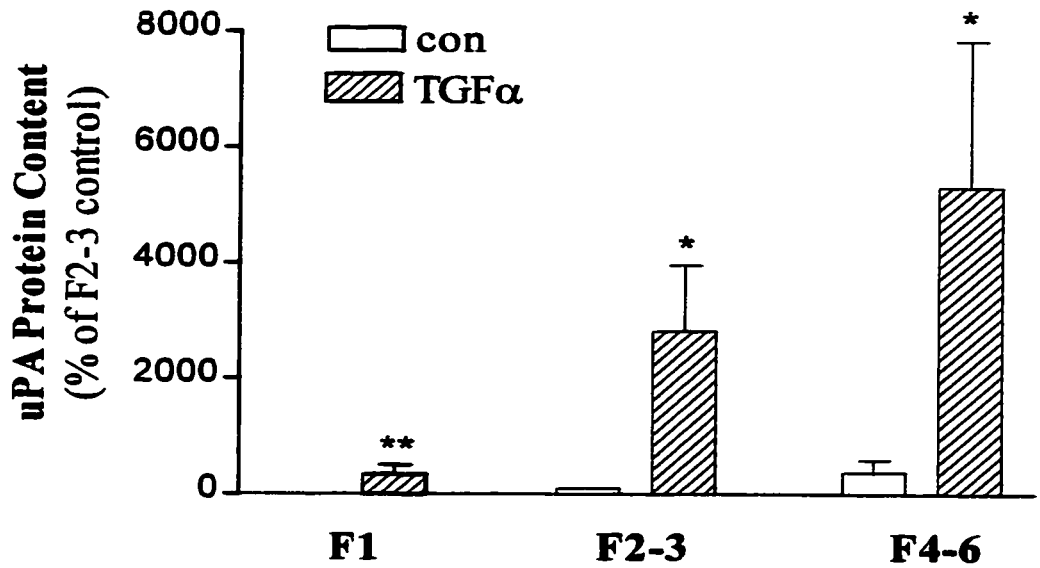

uPA mRNA Abundance
(% of F1 control)
30000
20000
10000
0
con
TGFα
*
**
F1
F2-3
F4-6

Figure 42: Influence of TGFα on uPA protein content in granulosa cells during follicular development. Granulosa cells from F1, F2-3 and F4-6 follicles were cultured for 21 h in the absence and presence of TGFα (10 ng/ml). Equal amounts of cellular protein (40-60 μg protein/lane, depending on specific experiment) was analyzed by Western blot. *Upper panel* shows a representative blot and *lower Panel* illustrates changes in uPA protein content as analyzed densitometrically, using the Image Analysis Systems from Bio-Rad Laboratories. Data represent mean±SEM of three experiments; "c" or "con", control; "α ", TGFα; *$p<0.05$, **$p<0.01$ (compared to respective controls)


F1
F2-3
F4-6
uPA
c
α
c
α
c
α


uPA Protein Content
(% of F2-3 control)
8000
6000
4000
2000
0
con
TGFα
**
*
*
F1
F2-3
F4-6

3. Influence of TGFα on granulosa cell PA activity

PA activity in cultured granulosa cells (PAc) and culture medium (PAm) were determined by fibrinolysis assay. The basal PAc activity was greater than that of PAm at all stages of follicular development (Table 3). Consistent with previous observations (Lafrance et al., 1993 a, b) and the changes in uPA transcript and protein levels noted in the present studies, total PA (PAc+PAm) activity likewise decreased with follicular maturation (ANOVA; $p<0.05$). TGFα also increased net PA activity at each follicular stage of development (Fig. 43).

Two isoforms of uPA are known to exist in various cell types: low (~35 kDa) and high (~50 kDa) molecular weight forms (Bachman, 1987; Dano et al., 1985; Saksela, 1985). To confirm fibrinolysis results and to determine the uPA isoform responsive to TGFα, PA activities in cultured granulosa cells at different stages of maturation were examined by fibrin overlay assay. Basal and TGFα-induced PA activities were associated with a molecular mass of about 35 kDa (Fig. 44). Consistent with the results observed by fibrinolysis assay, basal and TGFα-induced uPA activities were highest in granulosa cells from the less mature follicles (F4-6) and decreased with follicular maturation.

Figure 43: Effect of TGFα on net PA activity in granulosa cells during follicular development. Granulosa cells from F1, F2-3 and F4-6 follicles were cultured for 21 h in the absence (con) and presence of TGFα (10 ng/ml). Net total PA activities in cells plus medium were measured by fibrinolysis assay. Values represent means±SEM of seven replicate experiments. *$p < 0.05$ (compared to respective control)

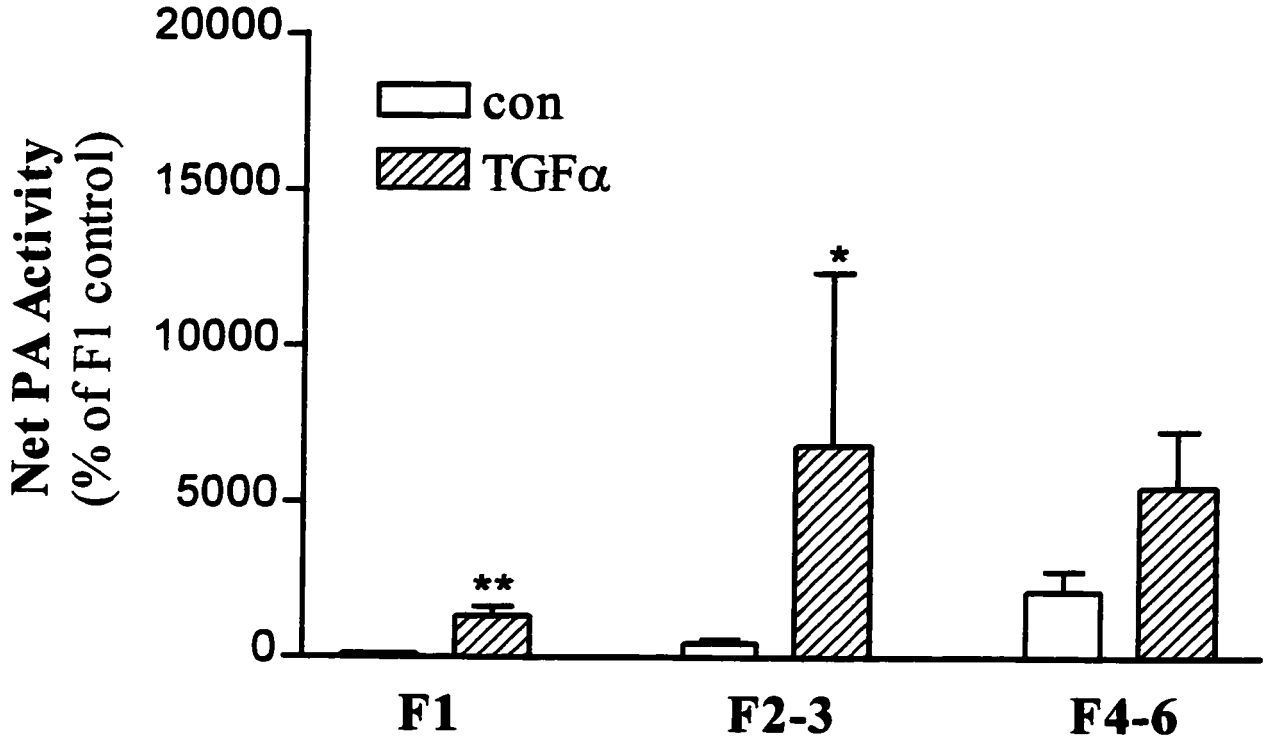
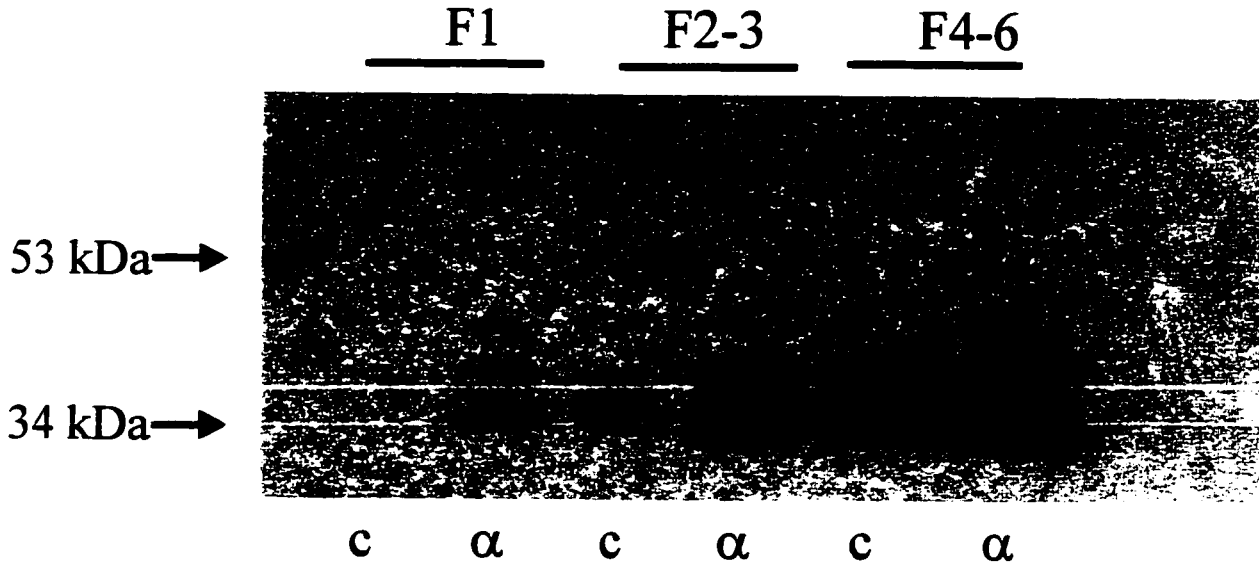

20000
15000
10000
5000
0
Net PA Activity
(% of F1 control)
con
TGFα
**
*
F1
F2-3
F4-6

Figure 44: Influence of TGFα on uPA activity in granulosa cells during follicular development. Granulosa cells from F1, F2-3 and F4-6 follicles were cultured for 21 h in the absence and presence of TGFα (10 ng/ml). Total PA activities (cells plus medium) were determined by the fibrin overlay assay. "c", control; "α ", TGFα.


F1
F2-3
F4-6
53 kDa
34 kDa
c
α
c
α
c
α

VIII. DISCUSSION

A. Regulation of PG production by TGFα and TGFβ during follicular development

The regulation of ovarian PG secretion by paracrine/autocrine factors is poorly understood. The present study provides the first demonstration that TGFα and TGFβ play an important role in the regulation of granulosa cell PG secretion. Whereas TGFα stimulated both basal PGF and PGE secretion by avian granulosa cells, TGFβ was inhibitory. The actions of these growth factors were concentration- and time- dependent. In the egg-laying hen, TGFα and TGFβ are produced by both theca and granulosa cells throughout follicular development (Onagbesan et al., 1994, Law et al., 1995). In addition, the expression of TGFα and its receptor (EGF receptor) is highest at the early stage of follicular development (Onagbesan et al., 1994), when the granulosa cells are proliferatively most active (Lafrance et al., 1993a&b) and more responsive to the growth factor (Li et al., 1994; Lafrance et al., 1993b). These observations raise the possibility that ovarian granulosa cell- and/or thecal-interstitial cell-derived TGFα and TGFβ have an autocrine and/or paracrine role in the regulation of PG secretion at the level of the granulosa cell during follicular development.

Previous studies by Tilly et al. (1992b) and Lafrance et al. (1993a & b) have demonstrated that cells from follicles at different stages of maturation respond to TGFα, while the less differentiated cells from F5-6 follicles also respond to TGFβ. The present study extends these observations and demonstrates not only the stimulatory effects of TGFα on PG secretion but also that the stimulation of PGs is more pronounced in cells from mature (F1, F3) follicles. In contrast, TGFβ suppressed basal and TGFα-induced PG secretion and the

inhibition was greatest in cells of the less mature follicles. The antagonistic action of TGFα and TGFβ observed in this study is consistent with previous reports (Dobson and Schombeng, 1987; Adashi and Resnick, 1986; Dorrington et al.,1988).

The ability of growth factors to influence PA activity has been documented (Lafrance et al., 1993a & b; Tilly and Johnson, 1990a&b). The results of the present study on granulosa cell PG secretion also indicate that TGFα stimulates avian granulosa cell PA activity during follicular development. In addition, TGFβ may function as a modulator of PA activity in response to TGFα at defined periods during follicular maturation. These observations are consistent with previous findings (Lafrance et al., 1993b) that transforming growth factors play important roles in the regulation of granulosa PA activity during follicular development. Moreover, the present data confirm that the TGFα-induced PA corresponded to uPA with a molecular weight of 35 KDa. Although the mechanism of action of TGFα in the regulation of granulosa cell PA activity in the hen remains to be determined, Lax et al.(1988) have shown that TGFα binds to the avian EGF receptor, a receptor type normally known to be associated with, or containing, tyrosine kinase.

There is no consensus regarding the relationship between the increases in cellular PG concentration and PA activities in the ovary. Beers et al. (1975) first demonstrated that there is a marked increase in PA activity in the rat ovary near the time of ovulation, which may be related to an increase in PG concentration. While PGE_1 and PGE_2, but not $PGF_{1\alpha}$ and $PGF_{2\alpha}$ have been shown to stimulate PA activity in rat granulosa cells (Strickland and Beers, 1976), the cyclooxygenase inhibitor indomethacin suppressed hCG- and GnRH agonist-induced PA secretion (Liu and Stephen 1988). In the domestic hen, PGE_2 has also been reported to

slightly (26%) but significantly increase granulosa cell PA activity (Tilly and Johnson, 1987). However, other studies concluded that PG are not involved in the preovulatory synthesis of PA induced in the rat ovary by hCG. This notion was based on the observation that indomethacin, which blocked ovulation, failed to inhibit ovarian PA activity (Shimada et al., 1983; Espey et al., 1985). The present study has shown that the response of PA activity to transforming growth factors is not related to granulosa cell PG secretion and action. This conclusion is supported by the following observations: a) basal PG secretion increased but basal PA activity decreased during follicular maturation, b) PG secretion in the presence of TGFα was more pronounced in cells from mature follicles, while PA activity was highest in cells from immature follicles, c) basal and TGFα-induced PG secretion were suppressed by TGFβ while, depending on the stage of follicular development, TGFα-induced PA activity was either not affected or enhanced by TGFβ, and d) indomethacin significantly inhibited TGFα-induced PG secretion but had no effect on TGFα-induced PA activity. Although our findings do not support an autocrine regulatory role of PG in hen granulosa cell PA, the possibility that PG from the theca cells may be important in regulating enzymes involved in follicular wall remodelling during late stages of follicular development cannot be excluded. The latter contention is consistent with the earlier observation that PGE_2 can increase PA activity in preovulatory folicle granulosa cells from F1 follicles *in vitro* (Tilly and Johnson, 1987).

In summary, we have shown that TGFα stimulated granulosa cell PG secretion and PA activity, whereas TGFβ inhibited PG secretion and had no effect on basal PA activity. The regulation of granulosa cell PG and the interaction of TGFα and TGFβ were dependent

on follicular maturation. The action of TGFα on uPA activity seems unrelated to the changes in PG secretion induced by the growth factors.

B. TGFα-induced granulosa cell DNA synthesis is mediated by PGs during follicular development

PG synthesis in granulosa cells during ovarian follicular development has recently been shown to be regulated by a complex interaction of TGFα and TGFβ (Li et al., 1994). The physiologic role of these eicosanoids in the control of ovarian cell function by these growth factors is unknown. The study in this section provides the first demonstration of an involvement of arachidonic acid metabolites in the regulation of DNA synthesis by growth factors in ovarian cells. Our conclusion that PGs are key elements in the mitogenic signalling pathway for TGFα in preovulatory follicle granulosa cells is supported by the following evidence: (i) TGFα stimulates PG production at concentrations similar to those required to promote DNA synthesis, (ii) TGFα-induced PGE and PGF production and DNA synthesis are suppressed by inhibitors of PLA_2 (ONO-RS-82) and cyclooxygenase (ibuprofen, naproxen), (iii) the inhibitory effect of ONO-RS-82 on DNA synthesis can be reversed by exogenous PGE_2, (iv) PGE_2 mimics the effect of TGFα on DNA synthesis in granulosa cells, and (v) PGs can potentiate the stimulation of DNA synthesis by a submaximal stimulatory concentration of TGFα. Our findings are consistent with a growing body of evidence suggesting PGs as central elements in cell signalling for mitogenesis induced by growth factors and in oncogenic transformation (Hakeda et al., 1987; Honn et al., 1981; Earnest et al., 1993). Increased PG production has been associated with many tumour cells and is believed to regulate tumour growth and metastasis (Honn et al., 1981). PGE_2 and $PGF_{2\alpha}$ have been shown to facilitate the mitogenic action of EGF in hepatocytes (Refsnes et al., 1994) and cytokines in B

lymphocytes (Garrone et al., 1994). In the present studies, we have demonstrated that, compared to PGs of the F series, PGE_1 and PGE_2 are more effective in augmenting TGFα-induced DNA synthesis in hen granulosa cells. Our findings, however, are at variance with the observations that $PGF_{2\alpha}$ is a more potent mitogen than PGE_2 in BALB/c3T3 fibroblasts (Nolan et al., 1988) and with those on pig tracheal smooth muscle cells where PGE_2 inhibited serum-induced thymidine incorporation (Florio et al., 1994). In addition, our studies indicate that neither the inhibitors of lipoxygenase nor exogenous leukotrienes significantly affected TGFα-induced DNA synthesis, while in Syrian hamster embryo fibroblasts and BALB/c3T3 fibroblasts, the products of this pathway are important in the mitogenic action of EGF (Nolan et al., 1988; Glasgow et al., 1992). Thus, it appears that cell-specific differences may exist in the role of PGs and leukotrienes in mitogenic regulation *in vitro*.

The present investigations with indomethacin have demonstrated that although the cyclooxygenase inhibitor markedly suppressed TGFα-stimulated PG production, it failed to significantly inhibit DNA synthesis induced by the growth factor *in vitro*. While the reason(s) for this apparent dichotomy is (are) not readily apparent, it is possible that the inhibitor has a non-specific effect on DNA synthesis, independent of its inhibitory action on PG synthesis. Our demonstration that indomethacin augmented the [^{3}H]thymidine incorporation into DNA, in granulosa cells stimulated by TGFα and PGE_2 supports this contention. This phenomenon exists also in Syrian hamster fibroblasts where PGE_2 markedly attenuated EGF-induced DNA synthesis, but indomethacin was ineffective in modulating the action of the growth factor (Glasgow et al., 1992).

The precise nature of PG involvement in the regulation of granulosa cell DNA synthesis by TGFα remains to be fully elucidated. Consistent with the DNA synthetic response to PGs, granulosa cell EP3 receptor expression was greater in earlier stages of follicular development. It has been suggested that activation of EP3 results in inhibition of adenylate cyclase (Yumoto et al., 1986b). There is no consensus regarding the role of cAMP in proliferation. PGE_2 has been reported to exert its mitogenic effect via elevating cellular cAMP levels in Swiss 3T3 cell, Swiss 3T6 mouse fibroblasts and human A431 epidermoid carcinoma cells (Huang et al., 1991). However, cAMP is antiproliferative in fibroblasts and human liver Ito cells (Mckenzie & Pouyssegur, 1996; Mallat et al., 1995). On the other hand, modulation of EGF-dependent DNA synthesis in Syrian hamster embryo cells by PGE_2 is associated with altered expression of c-jun and jun-B mRNA (Cowlen and Eling 1992). In addition, $PGF_{2\alpha}$ is a full mitogen in the BP-A31 and Swiss 3T3 cells and is believed to act via protein kinase C activation (Fagot et al., 1993; Garrone et al., 1993). PGFs bind to the FP and EP1 receptors coupled to G protein, activating phospholipase C (Haushka et al., 1989). Phospholipase C-mediated action of PGFs has been reported in MC 3T3-E1 cells, where stimulation of phosphatidyl inositol turnover and, in part, activation of protein kinase C were responsible for a mitogenic effect of $PGF_{2\alpha}$ (Hakeda et al., 1987). Interestingly, $PGF_{2\alpha}$ has also been demonstrated to increase free cytosolic calcium concentration in rat and human granulosa cells *in vitro* (Currie et al., 1992). Whether cAMP, Ca^{++} and protein kinase are indeed involved in the mitogenic action of PGs in ovarian cells remains to determined.

In the present study, PGs alone were capable of exerting only a weakly mitogenic

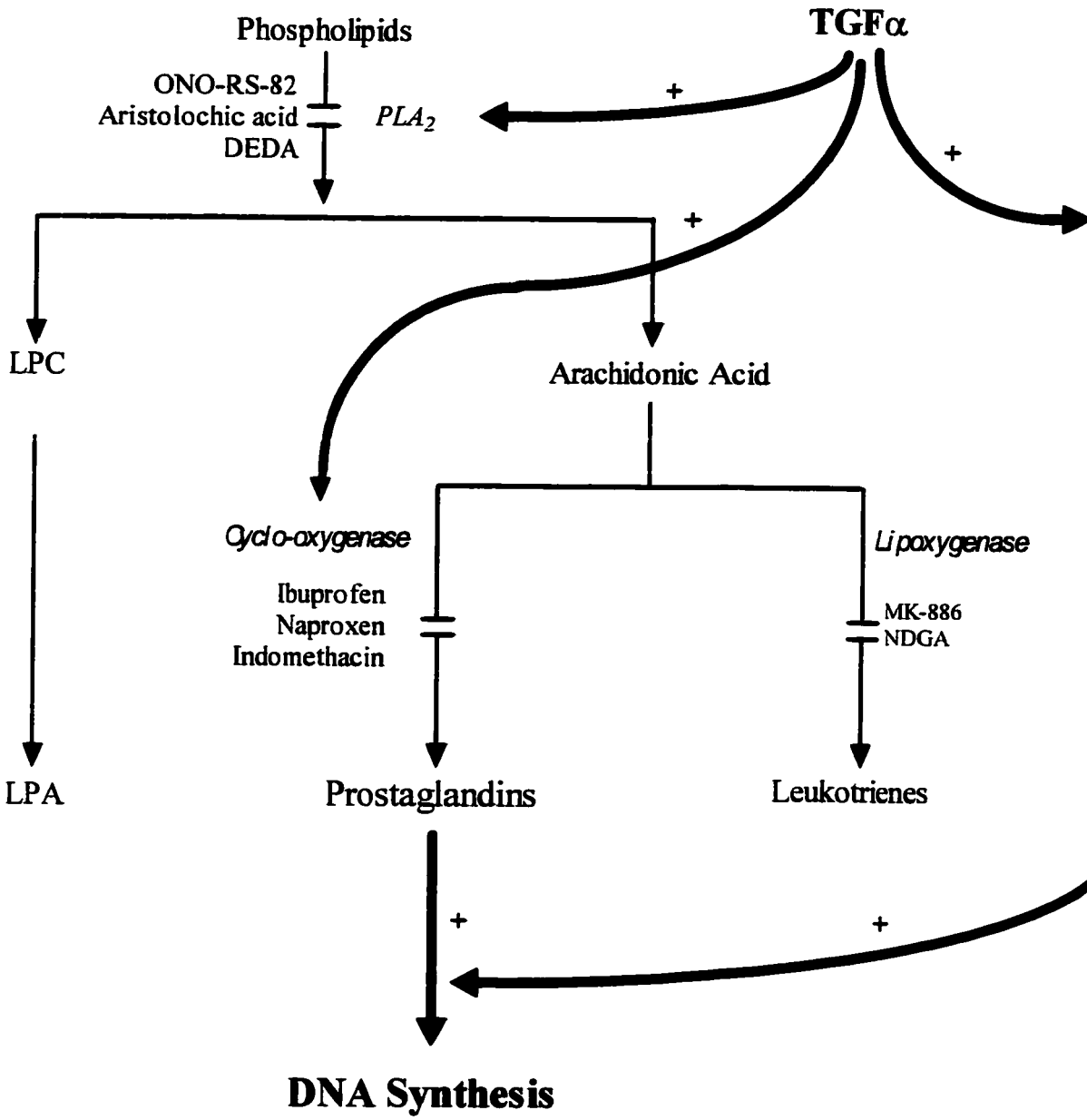
response in granulosa cells *in vitro*. However, when added to granulosa cell cultures in the presence of TGFα, PGs (especially PGE_2) were more effective in stimulating DNA synthesis. This response was particularly evident in granulosa cells from rapidly growing follicles (F5-6). This implies that the involvement of PGs is necessary but not sufficient in the regulation of granulosa cell DNA synthesis by TGFα, and that an undetermined factor "X" must be produced or activated to complete the signal transduction pathway for a full mitogenic response (Figure 45). Whether factor "X" represents an elevation of cytosolicIP_3 and/or Ca^{++} concentration, an increase of phosphatidylinositol 3' kinase activity or other events resulting from tyrosine kinase receptor activation requires further investigation.

Although TGFα has been suggested to play an important role in the regulation of ovarian follicular development (Lafrance et al., 1993a & b; Li et al.,1994), present knowledge on the mechanism by which the growth factor exerts its mitogenic influence on the granulosa cells during folliculogenesis is incomplete. The present studies demonstrate for the first time that PGs, particularly of the E series, may be important mediators in the action of TGFα in the regulation of granulosa cell proliferation during follicular development. This contention is based on the observations that the stimulation of DNA synthesis by exogenous PGE_2 (as that induced by the growth factor), as well as the ability of the PG to potentiate the mitogenic effect of a submaximal concentration of TGFα were more evident in F5-6 than F1 granulosa cells. In addition, the ability of PGE_2 to reverse the suppression of [^{3}H]thymidine incorporation into DNA by a PLA_2 inhibitor was greater in cells at earlier than later stage of follicular maturation. These follicular stage-dependent differences may be due to the greater abundance of EP3 receptors in early stages of

follicular development and programmed changes in production or activation of factor "X", as previously suggested (Hertelendy et al., 1989; Soboloff et al., 1995).

In summary, the present studies have demonstrated that the synthesis and action of PGs, particularly of the E series, are important events for the induction of DNA synthesis in hen granulosa cells by TGFα and that their involvement are dependent on the stage of follicular development. While these findings provide strong support for an important role of PG in the mechanism of action of TGFα in the regulation of cell proliferation during ovarian follicular maturation, an increase in PG synthesis/action is insufficient to fully express the mitogenic response of the growth factor. The identity and nature of involvement of factor "X" remains to be determined.

Figure 45: Hypothetical model of PG synthesis and action in granulosa cells.


Phospholipids
TGFα
ONO-RS-82
Aristolochic acid
DEDA
PLA2
+
+
+
LPC
Arachidonic Acid
Cyclo-oxygenase
Lipoxygenase
Ibuprofen
Naproxen
Indomethacin
MK-886
NDGA
LPA
Prostaglandins
Leukotrienes
+
+
DNA Synthesis

C. COX II is important in the signalling cascade for TGFα to regulate granulosa cell PG production and DNA synthesis during follicular development

Studies from this section demonstrate that COX II is regulated by TGFα and TGFβ at the transcriptional and translational levels in the hen ovary. The approximate size of the COX II transcript detected in the growth factor-stimulated hen granulosa cells (4.4Kb) was similar to that reported in chicken embryo fibroblasts (Xie et al., 1991) and in the rat ovary (Sirois et al., 1992). The increase in the abundance of COX II mRNA by TGFα was dependent on follicular development as a more rapid response to the growth factor was evident in F1 than in F5-6 granulosa cells (maximal response at : 4h vs 8h). Moreover, the transient nature of the response was noted by 12h in cells from F1 while COX II mRNA levels remained high in cells from less mature follicles even after 12 h of TGFα stimulation. Compared to the temporal response pattern of rat granulosa cells to gonadotropins, which peaked 4 h after hormonal challenge and almost disappeared at 6h (Gore-Langton and Armstrong 1988), high levels of COX II transcript induced by TGFα were sustained for much longer in hen granulosa cells (8h in F1; ≥12h in F5-6). It is not known whether this difference is due to the intrinsic characteristics of the agonists and/or species-specific differences in transcriptional activation, or the stability, of the COX II transcript. The 3'-untranslated region of the mRNA encoding COX II is known to contain many repeats of the pentanucleotide 5'-AUUUA-3', believed to be an important instability determinant in transcripts of proto-oncogenes (c-fos and c-myc) and cytokines (granulocyte-macrophage colony-stimulation factor, interferons) (Shaw and Kamen 1986; Schuler and Cole 1988). Therefore it is possible that the transient expression of COX II

mRNA may be partly determined by factors influencing its stability. The action of the growth factor on COX expression appeared to be specific for the type II isoform, since neither the presence of TGFα, the duration of growth factor exposure nor follicular maturity influenced granulosa cell COX I mRNA levels. The fact that no COX I transcript was detected when probed with chicken COX I cDNA suggests that the transcript was in low abundance or not expressed in the ovary, as has been reported in other species (Wong et al., 1989; Sirois et al., 1992).

In the current investigation, TGFα increased F1 granulosa cell COX II protein content in a time-dependent manner. This response was temporally associated with changes in the transcript level: maximal increase was observable at 6-12 h and remained elevated by 12 h of culture. In addition, the temporal response of the enzyme to TGFα was also associated with an increase in PG secretion which reached maximal level by 6 h. Whereas the percentage stimulation of PGF decreased at 12 h and thereafter, that of PGE remained constant throughout the culture period. While TGFα-stimulated PG secretion was higher in cells from mature follicle (F1), the magnitude of change in COX II mRNA abundance and protein content was not dependent on follicular maturation. As also observed in the maximal increase in mRNA abundance induced by TGFα between cells from F1 and F5-6, the magnitude in COX II protein response appeared to be independent on the stage of follicular development (i.e. COX II level similar in granulosa cells from both follicle sizes in absence and presence of the growth factor).

COX II was originally isolated from immediate-early gene products in mitogen-stimulated cells (Xie et al., 1991). This is of particular interest in light of the important role of PGs in the regulation of ovarian granulosa cell proliferation during folliculogenesis.

As shown previously, the synthesis and action of PGs are important events in the cascade for the induction of DNA synthesis by TGFα in hen granulosa cells. Like TGFα, PGs are more effective in stimulating DNA synthesis at an early stage than at late stage of follicular development (Li and Tsang, 1995). In addition, the role of PG in the regulation of granulosa cell differentiation has also been extensively reported and PGs of the E series are known to stimulate steroidogenesis in rat granulosa cells (Sirois et al., 1992). In the present studies, however, PG production following TGFα stimulation were higher at the late stage of follicular development when granulosa cell proliferation is relatively less active. Although the reason(s) for the apparent dichotomy is unknown, it is possible that PGs may play multiple roles in the regulation of both proliferation (early follicular development) and differentiation (late follicular development) of granulosa cells. The presence and abundance of specific PG receptor subtypes on granulosa cells during follicular maturation may also be important determinants of the precise role of these bioactive lipids.

Recent studies by Law et al. (1995) have demonstrated that TGFβ is expressed in hen granulosa and theca cells throughout follicular development. In the present investigation, TGFβ decreased both basal and TGFα-stimulated granulosa cell COX II mRNA abundance. Like the temporal stimulatory action of TGFα, this inhibition by TGFβ was also follicular stage-dependent, as its effect on both basal and TGFα-stimulated transcript level were greater in cells from less mature follicles. The antagonistic action of TGFα and TGFβ in the regulation of COX II and PG secretion provide further evidence that TGFβ is a modulator of TGFα action in the ovary during follicular maturation, which is consistent with previous observations in mammals (Dobson and Schomberg 1987;

Adashi and Resnick 1986; Dorrington et al., 1988).

The signal transduction pathways involved in the induction of COX II in the hen granulosa cells are not known. Although cAMP is believed to be the mediator in the gonadotropic control of COX II gene expression in rat granulosa cells, the regulation by GnRH may involve protein kinase C and/or tyrosine kinase (Wong and Richards 1992). In the latter context, a possible involvement of tyrosine phosphorylation was supported by the observations that the induction of COX II by these hormones was blocked by genistein (a potent inhibitor of tyrosine kinase), and that EGF was capable of increasing, though only to a modest extent, COX II mRNA abundance *in vitro* (Wong and Richards 1992). Defining the precise regulatory mechanism(s) involved in regulating the expression of this gene will be an important step towards understanding the role of PGs in granulosa cell proliferation and differentiation during follicular development.

In summary, the present study demonstrated that COX II is selectively stimulated by TGFα and suppressed by TGFβ in hen granulosa cells *in vitro*, suggesting that regulation of PG production by the growth factors is predominantly at the level of COX II. Whereas COX II may be important in the signalling cascade for TGFα in the regulation of granulosa cell proliferation, the follicular stage-dependent PG secretion response of the cells to the growth factor may be a consequence of other contributing factors including differences in phospholipase A2 activity and/or PG metabolism at different stages of follicular development.

D. The regulation of PG production by TGFα and TGFβ is also at the level of $cPLA_2$

In previous chapters, we have demonstrated that PG production in hen granulosa cells is stimulated by TGFα and suppressed by TGFβ (Li et al., 1994). In addition, our study has also indicated that one of the sites of this regulation is COX II (Li et al., 1996). Although TGFα-stimulation and TGFβ inhibition of PG secretion were dependent on follicular development, the magnitudes of COX II mRNA abundance and protein content in response to the growth factors were independent of follicular maturation, raising the possibility that other compounding factors may be involved in the follicular stage dependent regulation. Another rate-limiting step in the control of PG production is the release of AA from phospholipids by the action of PLA_2. Although $cPLA_2$ is highly specific for the release of AA and is physiologically regulated (Clark et al., 1995), the role and the control of this enzyme in the regulation of ovarian PG production by these growth factors remain to be determined. The data presented here provide the first evidence for the regulation of $cPLA_2$ by transforming growth factors in the ovary. In hen granulosa cells, $cPLA_2$ appears as a 100 kDa protein on SDS PAGE and has a transcript size of ~3.2 kb, similar to that reported in human monocytes and neutrophils (Nakamura et al., 1992; Nahas et al., 1996). Basal granulosa cell $cPLA_2$ protein level increases with follicular development, a follicular stage-dependent distribution pattern consistent with the basal production of PGE by granulosa cells during follicular maturation (F1 > F5-6). The control of $cPLA_2$ may be one of the mechanisms in place to ensure adequate amounts of AA available for PG production at the appropriate stage of follicular development.

Recent studies on the regulation of $cPLA_2$ have demonstrated that the enzyme migrates as a doublet and exhibits increased activity as well as decreased electrophoretic

mobility upon phosphorylation (Nakamura et al., 1992; Nahas et al., 1996). MAP kinase is believed to be involved in the phosphorylation of ser-505 of $cPLA_2$, which results in the alteration in the electrophoretic mobility of $cPLA_2$ and the activation of the enzyme (Lin et al., 1993). In the present study, challenge of hen granulosa cells with TGFα *in vitro* resulted in a shift of $cPLA_2$ from an electrophoretically fast to a slow migrating form. Pretreatment of the cells with inhibitors of serine/threonine kinase (staurosporin), MAP kinase kinase (MEK; PD98059) or MAP kinase (apigenin) effectively prevented the action of the growth factor. Although the precise mechanism(s) of $cPLA_2$ activation in granulosa cells by TGFα remains to be elucidated, it is tempting to speculate that the binding of TGFα to its receptor (EGF receptor) triggers a series of intracellular changes, including an increase in tyrosine kinase activity which activates MAP kinase, thereby resulting in phosphorylation of the serine residues of $cPLA_2$ and activation of the enzyme. Interestingly, stimulation of human neutrophils and platelets with granulocyte /macrophage colony stimulating factor (GM-CSF) and thrombin, respectively, resulted in increased MAP kinase activity and $cPLA_2$ phosphorylation and activation (Durstin et al., 1994; Kramer et al., 1991). In addition, treatment of platelet-activating factor-stimulated macrophage cell line $P338D_1$ with the tyrosine kinase inhibitor genistein suppressed both MAP kinase activation and arachidonate release (Glaser et al., 1990). Earlier studies from our laboratory have shown that the stimulation of granulosa cell PG production by TGFα is also sensitive to genistein (J. Li & B.K.Tsang, unpublished data), thus supporting a possible requirement for tyrosine phosphorylation in this regulation.

A considerable number of studies have focused on the upregulation of $cPLA_2$ by growth factors and cytokines (Bauldry et al., 1996; Kol et al., 1997a&b; Lin et al., 1993;

Nakamura et al., 1992; Nahas et al.,1996; Durstin et al., 1994; Hoeck et al., 1993; Chepenik et al., 1994). If $cPLA_2$, and thus PGs, indeed play an important role in physiological and pathological processes, one would expect that inhibitory mechanisms should be in place to prevent over-expression of $cPLA_2$, and thereby excessive PG production, to maintain a homeostatic state. Although the identity and nature of these mechanisms are yet to be demonstrated, results from the present study are consistent with this notion. In the absence of exogenous growth factors, granulosa cell $cPLA_2$ mRNA and protein content increased with length of culture. Although the precise mechanism for this increase is not known, one possibility is that granulosa cell $cPLA_2$ expression is suppressed by inhibitory factors (such as TGFβ) and their signalling pathways to maintain appropriate levels *in vivo*. When these inhibitory factors were "removed" as the cells were cultured in serum-free medium, $cPLA_2$ expression spontaneously increased. In addition, we have demonstrated that these increases in basal $cPLA_2$ expression and protein levels related to the duration of culture in the absence of exogenous growth factor were accompanied by reciprocal decreases in expression of MADR2 (Mother Against dpp Related Protein 2), a component of the TGFβ signalling pathway and a possible transcriptional regulator. In this context, our results here provided the first evidence that $cPLA_2$ is regulated by TGFβ. The decrease in granulosa cell $cPLA_2$ protein content by TGFβ was concentration- and time- dependent and appeared to be temporally associated with changes in $cPLA_2$ transcripts levels, as $cPLA_2$ mRNA abundance was likewise decreased by the growth factor in a time-dependent manner. Interestingly, whereas TGFβ alone had no effect on $cPLA_2$ transcripts and PG production in mouse embryonic cells, it potentiated the stimulatory action of EGF (Chepenik et al., 1994), indicating a species- or cell-specific difference in the response of $cPLA_2$ to the growth factor.

Defining the precise mechanism of regulation of $cPLA_2$ expression by TGFβ will be an important step towards understanding the complexity of the regulation of $cPLA_2$ and, thus of PG production during ovarian follicular development. The signal transduction pathway involved in the suppression of $cPLA_2$ by TGFβ in hen granulosa cells will be discussed in the next section.

Our previous study also demonstrated that one of the mechanisms for TGFα to stimulate PG production is via an increase in COX II mRNA level and protein content (Li et al., 1996). Although TGFβ markedly suppressed PG production in granulosa cells, it failed to decrease basal COX II protein content (Li et al., 1996). Therefore, the regulation of PG production by TGFα is at the levels of both $cPLA_2$ and COX II, while that by TGFβ is more important at the level of $cPLA_2$. In light of the fact that arachidonic acid is not only a precursor of eicosanoids but also a second messenger (Clark et al., 1995), the dual regulation by TGFα at both the level of COX II and $cPLA_2$ may be one of the mechanisms by which the growth factor elicits different physiological responses.

In summary, the present study demonstrated for the first time, the presence and regulation of $cPLA_2$ in the hen ovary. The finding that PLA_2 phosphorylated by TGFα and suppressed by TGFβ suggests that the control of PG production by the growth factors is also at the level of this enzyme. Moreover, although the positive regulation of $cPLA_2$ has been shown in a number of cellular systems (as in the granulosa cells by TGFα in the current report), the present studies represent the first demonstration of $cPLA_2$ down-regulation in a physiologic cell model. Cytosolic PLA_2 may be important in the signalling cascade for the growth factors in the regulation of granulosa cell proliferation during follicular development.

E. Importance of autoregulation of Mother Against dpp Related Protein 2 (MADR2) by TGFβ in conferring follicular-stage dependent suppression of granulosa cell $cPLA_2$ expression by the growth factor

Cell signalling by TGFβ is mediated by two types of transmembrane serine/threonine kinase receptors, type I and II (Kingsley 1994). The type II receptor appears to be constitutively active and, upon ligand binding, forms a heteromeric complex with type I receptor that activates the type I receptor by phosphorylation (Wrana et al., 1994). Recently, MAD and MAD-related proteins (MADR) have been identified in a variety of species as important components of the signal transduction pathway of the TGFβ superfamily (Attisano and Wrana 1996; Massague et al., 1996a; Graff et al., 1996). It has been demonstrated that MADR2 is rapidly phosphorylated by activation of TGFβ signalling pathway (Eppert et al., 1996) and is redistributed from the cytoplasm to the nucleus, presumably for transcriptional activation of specific genes (Niehrs 1996). In the present studies, we have demonstrated for the first time the presence of MADR2 protein (60KDa) and transcript (3.6 kb) in granulosa cells and, more importantly, its expression is up- regulated by TGFβ at both transcriptional and translational levels. This is evident by a time- and concentration- dependent increase in granulosa cell MADR2 mRNA abundance and protein content in the presence of TGFβ. These findings suggest that, in addition to being phosphorylated and activated by TGFβ receptor activation, as has been shown in other systems (Eppert et al., 1996), MADR2 is also up-regulated by TGFβ, and thus, presumably acts to enhance its own actions. Interestingly, as shown in the time course study, granulosa cell MADR2 transcript levels decreased with the duration of culture in growth factor-free conditions and could be reversed by the addition of exogenous TGFβ (Fig.39A&B), suggesting that the growth factor may be

necessary for the maintainence of physiologically important cellular levels of MADR2 *in vivo*.

Previous studies from our laboratory have demonstrated that TGFβ suppresses granulosa cell $cPLA_2$ protein expression and COX II mRNA abundance and prostaglandin production as well as increasing urokinase plasminogen activator activity *in vitro* and that these responses were more pronounced at early stages of follicular development (Li and Tsang 1995; Li et al.,1996; Lafrance et al., 1993b). In the present study, we have demonstrated that MADR2 is up-regulated by TGFβ *in vitro*, with maximum response observed in granulosa cells from F5-6 follicles. Whereas TGFβ is expressed in hen granulosa and theca cells throughout follicular development (Law et al., 1995), our present *in vivo* studies indicate that MADR2 abundance was minimal in granulosa cells from LWF, highest in F5-6 granulosa cells, and decreased with follicular maturation. These findings raise the interesting possibility that the follicular stage-specific high abundance of MADR2 may be an important determinant for the relative responsiveness of the signalling pathway to the growth factor, and that homologous up-regulation of MADR2 may be physiologically important. The physiological significance for the low MADR2 abundance in LWF granulosa cells observed in the present studies is not clear. It has been shown that TGFβ is capable of inducing apoptosis in another system (Mathieu et al., 1995). It is possible that the suppression of MADR2 expression *in vivo* at this follicular stage may be one of the mechanisms in place to prevent these cells from undergoing cell death until the fate of these follicles has been determined (i.e. development versus atresia).

MADR2 is believed to be a potential transcriptional regulator based on its ability to accumulate in the nucleus in response to TGFβ and its interaction with DNA-binding proteins. For instance, in Xenopus, Smad2 (MADR2) interacts with the forkhead-containing DNA-

binding protein, FAST1, at the activin responsive element in the *mix.2* gene (Chen et al., 1996b) Furthermore, MAD3, another member of MAD family, appears to be able to potentiate a transcriptional response toTGFβ following its overexpression (Chen et al.,1996a & b). However, the physiological target gene for MADR2 in the granulosa cell is not known, although it is tempting to speculate that the down-regulation of $cPLA_2$ by TGFβ may be related to the follicular stage- dependent expression of MADR2. This notion is consistent with our earlier observation that the suppression of granulosa cell $cPLA_2$ level by the growth factor resides at the level of gene transcription (Li et al., 1997). In addition, it is of interest to note from the present studies that alterations in MADR2 expression appear tightly coupled to reciprocal changes in $cPLA_2$ mRNA abundance, as evidenced by an increased basal MADR2 mRNA and protein level while $cPLA_2$ transcript abundance decreased with follicular development. In addition, basal MADR2 expression decreased while that of $cPLA_2$ increased with the duration of culture. Although the precise mechanism for the increase in $cPLA_2$ mRNA and protein content is not known, one possibility is that granulosa cell $cPLA_2$ expression is suppressed by endogenous inhibitory factors (e.g. TGFβ) and their signalling machinery to maintain appropriate levels *in vivo*. When these inhibitory factors were "removed" by culturing cells in serum-free medium, $cPLA_2$ expression spontaneously increased. This notion is consistent with our present observation that the elevation in basal $cPLA_2$ expression with increased duration of culture is accompanied by reciprocal decreases in mRNA abundance and protein content of MADR2. Finally, the reciprocal MADR2 and $cPLA_2$ response to TGFβ followed a similar time course, concentration- and follicular stage-dependency. Further experiments including MADR2 knock-out with antisense are required to directly determine whether and what precise relationship exists between MADR2 signalling

and $cPLA_2$ expression in the granulosa cell.

It has been demonstrated that MADR1 shares considerable sequence homology with MADR2 but is not involved in the TGFβ signalling pathway (Hoodless et al 1996). Although significant levels of MADR1 mRNA and protein were detected in granulosa cells in the present studies, they were not significantly affected by the presence of TGFβ, indicating that the response in MADR2 expression to the growth factor was specific.

In summary, we have demonstrated for the first time the presence of MADR1 and MADR2 in hen granulosa cells. The expression of MADR2, but not of MADR1, was up-regulated by TGFβ *in vitro* in a concentration- and time- dependent manner. Granulosa cell MADR2 expression was maximal during early stages of follicular development when the cells are proliferatively most active and the $cPLA_2$ system most responsive to TGFβ. These findings are consistent with the hypothesis that MADR2 expression is autoregulated, and that this regulation may be an important determinant in the follicular stage- specific responsiveness of the cells to TGFβ.

F. Urokinase plasminogen activator (uPA) is up-regulated by TGFα, during follicular development

Granulosa cell proliferation and ovarian follicular growth are accompanied by the dissolution of the surrounding cellular matrix and tissue remodelling, which may involve proteases such as uPA. TGFα stimulates uPA activities in avian granulosa cells during follicular development (Lafrance et al., 1993a; Tsafriri et al., 1989). This increase in uPA activity is accompanied by an increase in DNA synthesis (Lafrance et al., 1993a), indicating the possible association between the growth factor-induced cell proliferation and stimulation of uPA activity during follicular development. However, the precise mechanism by which TGFα stimulates uPA activity during follicular development remains to be defined. The present study demonstrates granulosa cell uPA is regulated at both mRNA and protein level by TGFα during follicular development in the avian ovary. The approximate size of the uPA transcript detected in basal and the growth factor-stimulated hen granulosa cells (2.5kb) is similar to that reported in chicken embryo fibroblasts (Bell et al., 1990) and in F1 granulosa cells (Tischkau et al., 1996). Consistent with the notion that uPA may be more important in ovarian functions requiring extracellular matrix degradation, such as granulosa cell proliferation and follicular growth, basal uPA mRNA abundance was greatest in granulosa cells from F4-6 and decreased with follicular maturation. TGFα significantly increased uPA mRNA levels, which reached maximum in proliferatively more active granulosa cells from follicles undergoing rapid growth, indicating that the regulation of uPA mRNA by TGFα may be dependent on follicular maturation. The precise mechanism(s) responsible for this increase in uPA transcripts is (are) unclear. Further studies including nuclear run-on assays and on the influence of TGFα on uPA mRNA half-life are required to determined whether

the regulation is at the transcriptional and/or post-transcriptional levels.

Consistent with the changes in uPA mRNA abundance, basal uPA protein content and activity were also highest in the less mature follicles (F4-6) and decreased with follicular maturation. Like uPA mRNA, uPA protein content and activity were maximal in granulosa cells from rapidly growing follicles, while the percentage stimulation by TGFα was lower than those in more mature follicles (e.g. PA activity in F4-6 vs F2-3 and F1: 2.5-fold vs 15- and 13- fold, respectively). Although the physiological significance of this phenomenan is not readily apparent, one explanation may be that, for homeostatic reasons, granulosa cell uPA protein and activities can only be up-regulated to a finite level so as to avoid premature follicular rupture. It is possible that basal PA activity in F4-6 granulosa cells has already been stimulated *in vivo* by endogenous TGFα, which is present at high levels at early stages of follicular development (Onagbesan et al., 1994), and no large increases could further be elicited by the growth factor *in vitro*. Indeed, preliminary findings from our laboratory have indicated high uPA mRNA abundance, protein content and activity in cultured granulosa cells from the small yellow follicles and failure of these parameters in these highly proliferative cells to respond to TGFα *in vitro* (Li et al; unpublished data). Interestingly, uPA is only detectable in granulosa cells from the less mature follicles from DES- treated immature rats (Karakji and Tsang 1995a, b, c & d). Though slightly stimulatory on basal uPA activity, TGFα suppressed the activity of the enzyme induced by FSH (Karakji and Tsang 1995a), indicating the species specific follicular stage-dependent uPA expression and response of granulosa cells to the growth factor.

Both the high and low molecular weight active forms of uPA have been identified in various vertebrate cell systems. These isoforms share an identical B-chain which contains

the active sites (Saksela et al., 1988) and differ in the molecular weight due to limited proteolytic cleavage of the 24 kDa A chain of the high molecular weight urokinase, leaving a 21 amino acid-polypeptide attached to the ~30 kDa chain (Saksela 1985). Western blot analysis in the current investigation identifies the basal and TGFα-stimulated uPA as a 35 kDa protein. These finding are consistent with our early observations that the basal and growth factor-induced PA activities in hen granulosa cells are associated with a molecular mass of ~35 kDa (Lafrance et al., 1993a) and suggest that this isoform of uPA may be the predominant form in granulosa cells of this species. Although only the 35 kDa form uPA was observed in the present study, using fibrin overlay assays, previous studies from our laboratory on hen granulosa cells also demonstrated that, in addition to the 35 kDa form, a ~50 kDa uPA was occasionally present in TGFα-stimulated granulosa cells (Lafrance et al., 1993a) , suggesting that the high molecular weight form of uPA can be expressed in this cell system.

Other studies have also suggested that, in addition to its role in tissue remodelling, the 20 kDa A chain of uPA contains an epidermal growth factor-like domain that mediates the binding of uPA to its receptor (Appella et al., 1987). Urokinase PA binding to its receptor results in the generation of an unspecified intracellular signal for a change in cell proliferative potential (Anichini et al., 1994) and stimulated mitogenesis (Loskutoff et al., 1988). Moreover, inhibition of endogenous production of uPA significantly suppressed cell proliferation in the human melanoma cell line (Kirchheimer et al., 1987). It is not clear whether the high basal and TGFα-stimulated uPA present in proliferatively active granulosa cells from immature follicles can also function as a mitogen, in addition to its role in extracellular matrix degradation during early follicular development.

In avian ovaries, TGFα is produced in both granulosa and theca cells and its receptor (EGF receptor) is expressed in granulosa cells in all stages of follicular development (Onagbesan et al., 1994). The enhancement of uPA mRNA, protein and activity in granulosa cells by TGFα may result from paracrine and autocrine actions of the growth factor. For instance, in addition to its regulation by the TGFα secreted from the granulosa cell itself, granulosa cell uPA may also be up-regulated by TGFα from the theca and other region of the granulosa cell layer (ie. germinal disc). Consistent with this notion are the observations that the levels of PA activity present in granulosa-theca cell co-cultures or ovarian follicular tissues are markedly higher than the sum of those of the individual cell types and that a TGFα-like factor is present in theca-conditioned medium capable of stimulating hen granulosa cell PA activities during follicular development (Wang et al., 1993; Jackson et al., 1994). Similar phenomena have also been observed in the regulation of granulosa cell DNA synthesis by a TGFα-like factor secreted from the germinal disc region of the follicle (Tischkau et al., 1993).

In summary, we have demonstrated that granulosa cells are capable of expressing uPA at all stages of follicular development. Basal and TGFα-stimulated uPA mRNA, protein abundance and activity were higher in granulosa cells at early stages of follicular development and decreased with follicular maturation. These findings are consistent with the concept that uPA plays an important role, not only as a proteolytic enzyme for extracellular matrix remodelling during early follicular development, but also possibly as a mitogen to stimulate granulosa cell proliferation in these rapidly growing follicles.

IX. CONCLUSIONS

The results from the studies in this thesis demonstrated that, in addition to the well established role of gonadotrophins in the regulation of ovarian follicular development, transforming growth factor α and β may be important intra-ovarian regulators of granulosa cell mitogenesis, and thus of follicular growth. Figure 46 illustrates a hypothetical model in which TGFα exerts its mitogenic action via stimulation of PG-mediated DNA synthesis, a process coupled with the expression of urokinase PA believed to be involved in ovarian tissue remodelling during folliculogenesis. It is proposed that granulosa cell PG production is up-regulated by TGFα and that the synthesis and action of PGs (particularly of the E series) are important elements in the biochemical cascade leading to granulosa cell DNA synthesis and proliferation. The present findings that TGFβ modulates the regulatory action of TGFα at the level of COX II and $cPLA_2$ have provided important insight into the complex nature of growth regulation by these intra-ovarian factors in the hen ovary.

Another important aspect of the current research relates to the follicular stage-dependent regulation of the granulosa cells by transforming growth factors and to the possible cell signalling mechanism(s) involved. As summarized in Figure 47, the DNA synthetic response of the granulosa cells to PG was greater in granulosa cells at an early stages of follicular development. Although both basal and TGFα-induced PG production was greater in cells from mature follicles, the follicular stage-dependent response to PG may have been due to an increased expression of PG receptors. Indeed, EP3 receptor expression in granulosa cells is highest at early stages of follicular maturation.

Basal and TGFα-stimulated PG production was suppressed by TGFβ. The suppression was greater in granulosa cells from early stages of follicular development. While

TGFα selectively increased both COX II mRNA abundance and protein content, and appeared to activate $cPLA_2$ via a MAP kinase-dependent pathway, the follicular stage-dependent regulation of PG production by TGFβ appeared to be at the level of $cPLA_2$, since suppression of granulosa cell $cPLA_2$ expression by the growth factor was greater at early stages of follicular development.

Interestingly, MADR2, a signalling component of TGFβ, was expressed and selectively up-regulated by the growth factor in granulosa cells in a follicular stage-dependent manner, suggesting that the greater response of the cells to TGFβ during early follicular development was due to greater cellular abundance of MADR2. Our findings have also demonstrated that increased granulosa cell MADR2 expression in response to TGFβ may be associated with a decrease in $cPLA_2$ expression. MADR2 knockout experiments will further clarify if homologous up-regulation of MADR2 in granulosa cells is indeed an important determinant in follicular stage-specific responsiveness to TGFβ and possibly in the suppression of $cPLA_2$ gene transcription by the growth factor.

Lastly, TGFα-induced granulosa cell DNA synthesis was accompanied by activation of tissue remodelling process, since both basal and TGFα-induced PA activity were greater in proliferatively active granulosa cells at early stages of follicular development. Urokinase PA was regulated by TGFα at the protein and mRNA levels. The increase in PA activity in response to TGFα was, however, independent of PG production and action, suggesting the coupling of the mitogenic and tissue remodelling responses during follicular development do not involve this group of eicosanoids.

Figure 46: Hypothetical model of granulosa cell PG synthesis and action in response to growth factors.

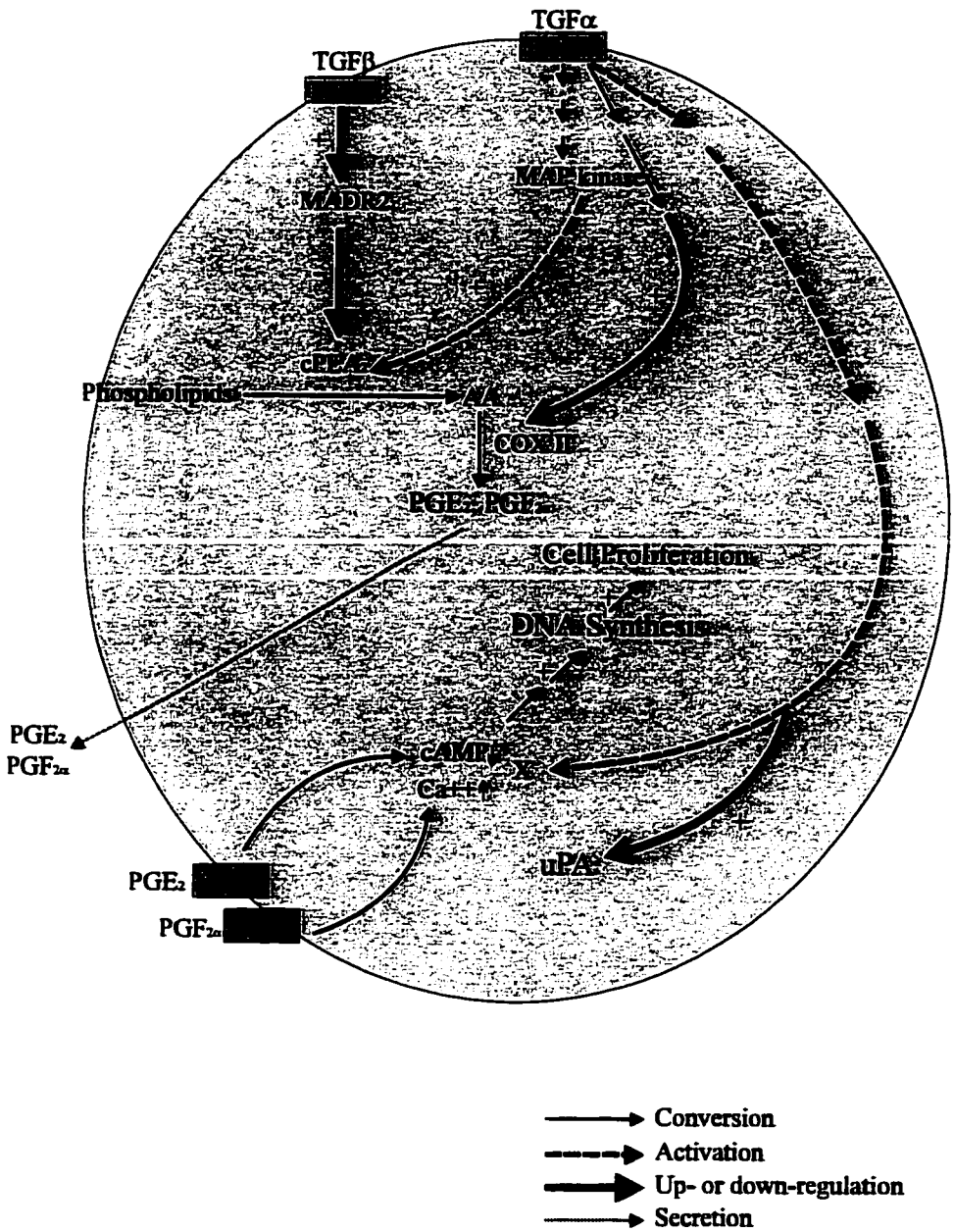
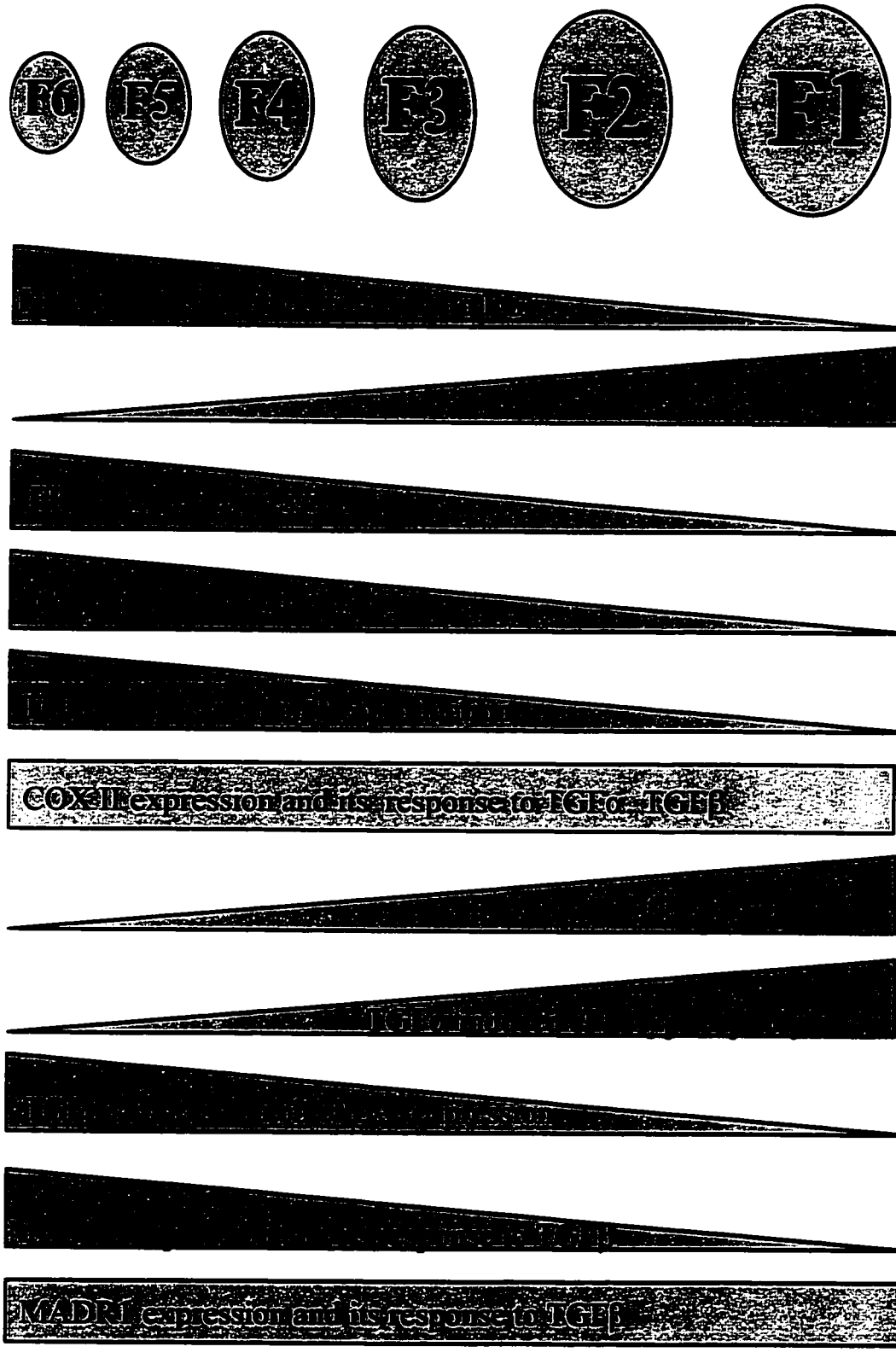

TGFα
TGFβ
MAPkinase
MADR2
cPLA2
Phospholipids
AA
COXIIE
PGE2 PGF2α
Cell Proliferation
DNA Synthesis
PGE2
PGF2α
cAMP
Ca++
uPA
PGE2
PGF2α
Conversion
Activation
Up- or down-regulation
Secretion

Figure 47: Dynamic changes of PG synthetic components, DNA synthesis, PA activity and their responses to growth factors in granulosa cells during follicular development.


F6
F5
F4
F3
F2
F1
COX-II expression and its response to IGE or TGFβ
MADR1 expression and its response to IGFβ

X. REFERENCES

Adashi EY 1992a The potential relevance of cytokines to ovarian physiology. J Steroid Biochem Mol Biol 43:439.

Adashi EY 1992b Intraovarian peptides, stimulators and inhibitors of follicular growth and differentiation. In: Endocrinology and Metabolism Clinics of North America. Kamran and Moghissi (eds). Saunders, Philadelphia 21:1

Adashi EY, Resnick CE 1986 Antagonistic interactions of transforming growth factors in the regulation of granulosa cell differentiation. Endocrinology 119:1979

Adashi EY, Resnick C, Croft C, Payne D 1989 Tumor necrosis factor α inhibits gonadotropin hormonal action in nontransformed ovarian granulosa cells. J Biol Chem 264:11591

Adashi EY, Resnick CE, Packman JN, Hurwitz A, Payne DW 1990 Cytokine-mediated regulation of ovarian function: tumor necrosis factor alpha inhibits gonadotropin-supported progesterone accumulation by differentiating and luteinized murine granulosa cells Am J Obstet Gynecol 162: 889

Ailenberg M, McCabe D, Fritz IB 1990 Androgens inhibit plasminogen activator activity secreted by sertoli cells in culture in a two chambered assembly. Endocrinology 126:1561

Ainsworth L, Tsang BK, Downey BR, Marcus GJ, Armstrong DT 1979 Effect of indomethacin on ovulation and luteal function in gilts. Biol Reprod 21:401

Andrade-Gordon P, Strickland S 1986 Interaction of heparin with plasminogen activators and plasminogen: effects of the activation of plasminogen. Biochem 25: 4033

Anichini E, Fibbi G, Pucci M, Caldini R, Chevanne M, Del Rosso M 1994 Production of second messengers following chemotactic and mitogenic urokinase-receptor interaction in human fibroblasts and mouse fibroblasts transfected with human urokinase receptor. Exp Cell Res 213:438-448.

Appella E, Robinson SA, Ullrich SJ, Stoppelli MO, Corti A, Cassani G, Blasi F 1987 The receptor-binding sequence of urokinase. A biological function for the growth-factor module of protease. J Biol Chem 262:4437

Armstrong JA, Tilly JL, Johnson AL 1990 Evidence for the presence of both a urokinase, tissue-type plasminogen activator (PA) in preovulatory follicles from the domestic hen. 23rd Annual Meeting of the Society for the Study of Reproduction, Knoxville TN, p 158 (abstract)

Asem EK, Carnegie JA, Tsang BK 1992 Fibronectin production by chicken granulosa cells *in vitro*: effect of follicular development. Acta Endocrinol 127:466

Asem EK, Zaker T, Bieller HV, Hertelendy F 1984 Progeserone production in granulosa cells of the domestic fowl, Effects of incubation media, pH, cell density and some other factors. Dom Anim Endocrinol 1:325

Astedt B, Lecander I, Brodin T, Lundblad A, Low K 1985 Purification of a specific placental plasminogen activator inhibitor by monoclonal antibody and its complex formation with plasminogen activator. Thromb Haemost 53:122

Attisano L, Wrana JL 1996 Signal transduction by members of the transforming growth factor-β superfamily. Cytokine and Growth Factor Reviews 7:327

Bachmann F 1987 Fibrinolysis. In: Thrombosis and Haemostasis. Verstraete M, Vermylen J, Lijnen HR, Arnout A (eds). International Society on Thrombosis and Haemostasis and Leuven University Press, Leuven pp 227

Baker TG 1963 A quantitative and cytological study of germ cells in the human ovaries. Proc R Soc London Ser B 158:417

Baker JC, Harland RM 1996 A novel mesoderm inducer, MADR2, functions in the activin signal transduction pathway. Genes Dev 10:1880

Bauldry SA, Wooten RE, Bass DA 1996 Activation of cytosolic phospholipase A_2 in permeabilized human neutrophils. Biochim Biophysica Acta 1299:223

Beaumont HM, Mandl AM 1962 A quantitative and cytological study of oogonia and oocytes in the foetal and neonatal rat. Proc R Soc London Ser B 155:557

Beers WH, Strickland S, Reich E 1975 Ovarian plasminogen activator: relationship to ovulation and hormonal regulation. Cell 6:387

Behrman HR, Romero RJ 1991 Prostaglandins and prostaglandin-like products in reproduction: eicosanoids, peroxides and oxygen radicals. In: Reproductive Endocrinology. Yen SSC, Jaffe RB (ed). Toronto:W.B. Saunders Company, pp 238

Belin D, Vassalli JD, Combepine C, Godeau F, Nagamine Y 1985 Cloning nucleotide sequencing and expression of cDNAs encoding mouse urokinase-type plasminogen activator. Eur J Biochem 148:225

Bell SM, Brackenbury RW, Leslie ND, Degen JL 1990 Plasminogen activator gene expression is induced bythe *src* oncogene product and tumor promotors. J Biol Chem

265:133

Bendell JJ, Dorrington JH 1990 Epidermal growth factor influences growth and differentiation of rat granulosa cells. Endocrinology 127:533

Bendell JJ, Lobb DK, Chuma A, Gysler M, Dorrington JH 1988 Bovine thecal cells secrete factor(s) that promote granulosa cell proliferation. Biol Reprod 38:790

Billig H, Furuta I, Hsueh AJW 1993 Estrogens inhibit and androgens enhance ovarian granulosa cell apoptosis. Endocrinology 133:2204

Billig H, Furuta I, Hsueh AJW 1994 Gonadotropin-releasing hormone directly induces apoptotic cell death in the rat ovary: biochemical and *in situ* detection of deoxyribonuleic acid fragmentation in granulosa cells. Endocrinology 134:245.

Blasi F, Behrendt N, Cubellis MV, Ellis V, Lund LR, Masucci MT, Moller LB, Olson DP, Pedersen N, Ploug M, Ronne E, Bano K 1990 The urokinase receptor and regulation of cell surface plasminogen activation. Cell Differ Dev 32:247

Boonstra J, Rijken P, Cremers F, Verkleij A, ven Bergen en Henegouwen P 1995 The epidermal factor. Cell Biol Intern 19:413

Boone DL, Yan W, Tsang BK 1995 Identification of a deoxyribonuclease I-like endonuclease in rat granulosa and luteal cell nuclei. Biol Reprod 53:1057

Boone DL, Tsang BK 1997 Identification and localization of a deoxyribonuclease I in the rat ovary. Biol Reprod 57:813

Brannstrom M, Wang L, Norman RJ 1993 Effects of cytokines on prostaglandins production and steroidogenesis of incubated preovulatory follicles of the rat. Biol Reprod 48:165

Buck PA, Schomberg DW 1988 [^{125}]Iodo-epidermal growth factor binding and mitotic responsiveness of porcine granulosa cells and modulated by differentiation and follicle-stimulating hormone. Endocrinology 122:28

Busso N, Huarte J, Vassalli JD, Sappino AP, Bellin D 1989 Plasminogen activator in mouse mammary gland. J Biol Chem 64:7455

Cajander SB, Hugin MP, Kristensen P, Hsueh AJW 1989 Immunohistochemical localization of tissue-type plasminogen activator in ovaries before and after induced and spontaneous ovulation. Cell Tissue Res 257:1

Carpenter G, Cohen S 1990 Epidermal growth factor. J Biol Chem 265: 7709-7712

Cesarone CF, Bolognesi C, Santi L 1979 Improved microfluorometric DNA determination in biological material using 33258 Hoechst. Anal Biochem 100:188

Chen HL, Marcinkiewicz JL, Sanchelo-Tello M, Hunt JS, Terranova PF 1993 Tumor necrosis factor-α gene expression in mouse oocytes and follicular cells. Biol Reprod. 48:707

Chen Y, Lebrun J-J, Vale W 1996a Regulation of transforming growth factor β- and activin-induced transcription by mammalian Mad proteins. Proc Natl Acad Sci USA

Chen X, Rubock MJ, Whitman M 1996b A transcriptional partner for MAD proteins in TGFβ signalling. Nature 383:691

Chepenik KP, Diaz C, Jimener SA 1994 Epidermal growth factor coordinately regulates the expression of prostaglandin G/H synthase and cytosolic phospholipase A_2 genes in embryonic mouse cells. J Biol Chem 269:21786

Chomczynski P, Sacchi N 1987 Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156

Chun SY, Eisenhauer K, Minami S, Billing H, Perlas E, Hsueh AJW 1996 Hormonal regulation of apoptosis in early antral follicles: follicle-stimulating hormone as a major survival factor. Endocrinology 137:1447

Clark JD, Schievella AR, Nalefski EA, Lin LL 1995 Cytosolic phospholipase A_2. J Lipid Mediators and Cell Signalling 12:83

Cowlen MS, Eling TE 1992 Modulation of c-jun and jun-B messenger RNA and inhibition of DNA synthesis by prostaglandin E_2 in Syrian hamster embryo cells. Cancer Res 52:6912

Croze F, Kennedy TG, Schroedter IC, Friesen HG 1990 Expression of rat prolactin-like protein B in deciduoma of pseudopregnant rat and in decidua during early pregnancy. Endocrinology 127: 2665-2672

Currie WD, Li W, Baimbridge KG, Yuen BH, Leung PCK 1992 Cytosolic free calcium increased by prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) gonadotropin-releasing hormone, and angiotensin II in rat granulosa cells and $PGF_{2\alpha}$ in human granulosa cells. Endocrinology 130:1837

Dano K, Andreasen PA, Grondahl-Hansen J, Kristensen P, Nielsen LS, Skriver L 1985 Plasminogen activators, tissue degradation, and cancer. Adv Cancer Res 44:139

Darbon JM, Oury F, Laredo J, Bayard F 1989 Tumor necrosis factor-alpha inhibits follicle-stimulating hormone-induced differentiation in cultured rat granulosa cells. Biochem Biophys

Res Commun 163:1038

Davis JS, Weakland LL, Weiland DA, Farese V, West LA 1987 $PGF_{2\alpha}$ stimulates phosphatidylinositol 4,5-bisphosphate hydrolysis and mobilizes intracellular Ca^{++} in bovine luteal cells. Proc Natl Acad Sci USA 84:3728

Degen SJF, Rajput B, Reich E 1986 The human tissue plasminogen activator gene. J Biol Chem 261:6972

de Haas GH, Postema NM, Nieuwenhuizen W,van Deen LM 1968 Purification and properties of an anionic zymogen of phospholipase A2 from porcine pancreas. Biochim Biophys Acta 159:118-129

Derynck R, Roberts AB, Winkler Me, Chen EY, Goeddel DV 1984 Human transforming growth factor alpha: precursor structure and expression in *E. Coli*. Cell 38:287

Deutch DG, Mertz ET 1970 Plasminogen: purification from human plasma by affinity chromatography. Science 170: 1095

Dobson WC, Schomberg DW 1987 The effect of transforming growth factor β on follicle-stimulating hormone-induced differentiation of cultured rat granulosa cells. Endocrinology 120:512

Dorrington J, Chuma AV, Bendell JJ 1988 Transforming growth factor β and follicle-stimulating hormone promote rat granulosa cell proliferation. Endocrinology 123:353

Durieux ME, Lynch KR 1993 Signalling properties of lysophosphatidic acid. TIPS 14:249

Durstin M, Dustin S, Molski TFP, Becker EL and Sha'afi RI 1994 Cytoplasmic phospholipase A_2 translocates to membrane fraction in human neutrophils activated by stimuli that phosphorylate mitogen-activated protein kinase. Proc Natl Acad Sci USA 91:3142

Earnest DL, Hixson LJ, Alberts DS 1993 Piroxicam and other cyclooxygenase inhibitors potential for cancer chemoprevention. J Cell Biochem (suplement) 161:156

Emoto N, Baird A 1988 The effect of tumor necrosis factor/cachectin on follicle-stimulating hormone-induced aromatase activity in cultured rat granulosa cells. Biochem Biophys Res Commun 153:792

Eppert K, Scherer SW, Ozcelik H, Pirone R, Hoodless P, Kim H,, Tsui LC, Bapat B, Gallinger S, Andrulis IL, Thomsen GH, Wrana JL, Attisano L 1996 MADR2 maps to

18q21 and encodes a TGFβ-regulated MAD- related protein that is functionally mutated in colorectal carcinoma. Cell 86:543

Erickson GF, Hsueh AJW 1978 Stimulation of aromatase activity by follicle stimulating hormone in rat granulosa cells *in vivo* and *in vitro*. Endocrinology 102:1275

Erickson GF 1986 An analysis of follicular development and ovum maturation. Semin in Reprod Endocrinol 4:233

Espey L, Shimada H, Okamura H 1985 Effect of various agents on ovarian plasminogen activator activity during ovulation in pregnant mare's serum gonadotropin-primed immature rats. Biol Reprod 32:1087

Evans CA, Kennedy TG, Patrick JE, Challis JRG 1981 Uterine prostaglandin concentration in sheep during late pregnancy and adrenocorticotropin-induced labour. Endocrinology 109:1533

Fagot D, Buquet-Fagot C, Mester J 1993 Mitogenic signaling by prostglandins in chemically transformed mouse fibroblasts: comparison with phorbol esters and insulin. Endocrinology 132:1729

Fenwick L, Jones RL, McGuire JC 1977 Production of prostaglandins by the pseudopregnant rat uterus *in vitro* and the effect of tamoxifen with the identification of 6-keto-$PGF_{1\alpha}$ as a major product. Br J Pharmacol 59:191

Ferguson EL, Anderson KV. 1992 Decapentaplegic acts as a morphogen to organize dorsal-ventral pattern in the Drosophila embryo. Cell 71:451

Fisher R, Waller EK, Grossi G, Thompson D, Tizard R, Schleuning WD 1985 Isolation and characterization of the human tissue-type plasminogen activator structural gene including its 5' flanking region. J Biol Chem 260:11223

Florio C, Martin JG, Styhler A, Helisler S 1994 Antiproliferative effect of prostaglandin E_2 in cultured guinea pig tracheal smooth muscle cells. Am J Physiol 266:L131

Ford KA, LaBarbera AR 1988 Follicle-stimulating hormone (FSH) unmasks specific high affinity FSH-binding sites in cell-free membrane preparations of porcine granulosa cells. Endocrinology 123:2374.

Fukuoka M, Yasuda K, Taii S, Mori T 1992 Synergistic actions of cytokines and growth factors in enhancing porcine granulosa cell growth. Endocrinol Jap 39:277

Gaddy-Kurten D, Tsuchida K, Vale W 1995 Activins and the receptor serine kinase

superfamily. Rec Prog Horm Res 50:109

Galway AB, Oikawa M, Ny T, Hsueh AJW 1989 Epidermal growth factor stimulates tissue plasminogen activator activity and messenger ribonucleic acid levels in cultured rat granulosa cells: mediation by pathways independent of protein kinase-A and -C. Endocrinology 125:126

Garrone P, Galibert L, Rousset F, Fu SM, Banchereau J 1994 Regulatory effects of prostaglandin E2 on the growth and differentiation of human B lymphocytes activated through their CD40 antigen. J Immunol 152:4282

Genton C, Kruithof EKO, Schleuing WD 1987 Phorbol ester induces the biosynthesis of glycosylated and non-glycosylated plasminogen activator inhibitor-2 in high excess over urokinase-type plasminogen activator in human U937 lymphoma cells. J Cell Biol 104:705

Gething MJ, Adler B, Boose JA, Gerard RD, Madison EL, McGookey D, Meidell RS, Roman LM 1988 Variants of human tissue-type plasminogen activator that lack specific structural domains of the heavy chain. Eur Mol Biol Organ J 7:2731

Gilbert AB 1979 Female genital organs. In: "Form and Function in Bird", Vol.1, Chapter 5. King AS and McLelland J (ed). London and New York: Academic Press.

Glaser KB, Asmis R and Dennis EA 1990 Bacterial liposacchride priming of P388D1 macrophage-like cells for enhanced arachidonic acid metabolism. J Biol Chem 265:8658

Glasgow WC, Afshari CA, Barrett JC, Eling TE 1992 Modulation of the epidermal growth factor mitogenic response by metabolites of linoleic and arachidonic acid in Syrian hamster embryo fibroblasts. J Biol Chem 267:10771

Goin M, Pifnataro O, Asua LJ 1993 Early cell cycle diacylglycerol (DAG) content and protein kinase C (PKC) activity enhancement potentiates prostaglandin F2α ($PGF_{2\alpha}$) induced mitogenesis in Swiss 3T3 cells. FEBS Letters 316:68

Gore-Langton RE, Armstrong DT 1988 Follicular steroidogensis and its control. In: The Physiology of Reproduction, vol 1.Knobil E, Neill JD (ed). Raven Press, New York, pp 331

Gospodarowicz D, Bialecki H 1979 Fibroblast and epidermal growth factors are mitogenic agents for cultured granulosa cells of rodent, porcine and human origin. Endocrinology 104:757

Gottshall PE, Katsuura G, Hoffmann ST, Arimura A 1988a Interleukin-1: an inhibitor of luteinizing hormone receptor formation in cultured rat granulosa cells. FASEB J 2:2492.

Gottshall PE, Katsuura G, Arimura A 1988b Interleukin-1 suppresses follicle stimulating hormone-induced estradiol secretion from cultured ovarian granulosa cells. J Reprod Immunol 15:281.

Gottshall PE, Uehara A, Hoffmann ST, Arimura A 1987 Interleukin-1 inhibits follicle stimulating hormone-induced differentiation in rat granulosa cells. Biochem Biophys Res Commun 149:502.

Graff JM, Bansal A, Melton DA 1996 Xenopus MAD proteins transduce distinct subsets signals for the TGFβ superfamily. Cell 85:479

Granelli-Piperno A, Reich E 1978 A study of proteases and protease-inhibitor complexes in biological fluids. J Exp Med 148:223

Gunzler WA, Steffens GJ, Otting F, Kim SMA, Frankus E, Flohe L 1982 The primary structure of high molecular mass urokinase from human urine. Hoppe-Seyler's Z. Physiol Chem 363:1155

Gurdon JB 1987 Embryonic induction, molecular prospects. Development 99:285

Hahn SA, Schutte M, Hoque AT, Moskaluk CA, da Costa LT, Rozenblum E, Weinstein CL, Fisher A, Yeo CJ, Hruban RH, Kern SE 1996 DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1. Science 271:350-353.

Hakeda Y, Hotta T, Kurihara N, Ikeda E, Maeda N, Yagya Y, Kumegawa M 1987 Prostaglandin E_1 and $F_{2\alpha}$ stimulate differentiation and proliferation, respectively, of clonal osteoblastic MC 3T3-E1 cells by different second messengers *in vitro*. Endocrinology 121:1966

Hamberg M, Svensson J, Samuelsson B 1975 Thromboxanes: A new group of biologically active compounds derived from prostaglandin endoperoxides. Proc Natl Acad Sci USA 72:2994

Hammond RW, Olson DM, Frenkel RB, Biellier HV, Hertelandy F 1980 Prostaglandins and steroid hormones in plasma and ovarian follicles during the ovulation cycle of the domestic hen. Gen Comp Endocrinol 42:195

Handler JA, Danilowicz RM, Eling TE 1990 Mitogenic signaling by epidermal growth factor (EGF), but not platelet-derived growth factor, requires arachidonic acid metabolism in BALB/c3T3 cells. J Biol Chem 265:3669

Harris RC, Homma T, Jacobson HR, Capdevia J 1990 Epoxyeicosatrienoic acids activate

Na^+/H^+ exchange and are mitogenic in cultured rat glomerular mesangial cells. J Cell Physiol 144:429

Haushka PV, Mais DE, Mayeux PR, Morinelli TA 1989 Thromboxane, prostaglandin and leukotriene receptors. Annu Rev Pharmacol Toxicol 29:213

Hawley SH, Wunnenberg-Stapleton K, Hashimoto C, Laurent MN, Watabe T, Blumberg BW, Cho KW 1995 Disruption of BMP signals in embryonic Xenopus ectoderm leads to direct neural induction. Genes Dev. 9:2923

Hay MF, Cran DG, Moor RM 1976 Structural changes occurring during atresia in sheep ovarian follicles. Cell Tissue Res 169:515

He CS, Wilhelm SM, Pentland AP, Marmer BL, Grant GA, Eisen AZ, Goldberg GI 1989 Tissue cooperation in a proteolytic cascade activating human interstitial collegenase. Pro Natl Acad Sci USA 86: 2632

Heaton JH, Gelehrter TD 1990 Cyclic nucleotide regulation of plasminogen activator and plasminogen activator-inhibitor mRNAs in rat hepatoma cells. Mol Endocrinol 4:171

Hedin L, Gaddy-Kurten D, Kurten R, DeWitt DL, Smith WL, Richards JS 1987 Prostaglandin endoperoxide synthase in rat ovarian follicles: content, cellular distribution, and evidence for hormonal induction preceding ovulation. Endocrinololgy 121:722

Heinrikson RL, Krueger ET, Keim PS 1977 Amino acid sequence of phospholipase A2-α from the venom of Crotalus adamanteus. A new classification of phospholipase A2 based upon structural determinants. J Biol Chem 252:4913

Hernandez ER, Twardzik DR, Purchio A, Adashi EY 1987 Gonadotropin dependent ovarian transforming growth factor beta gene expression. Biol Reprod 36 suppl 1:58

Hertelendy F, Nemecz G, Molnar M 1989 Influence of follicular maturation on luteinizing hormone and guanosine 5'o-thiotriphosphate-promoted breakdown of phosphoinositides and calcium mobilization in chicken granulosa cells. Biol Reprod 40:1144

Hertelendy F, Biellier HV 1978 Evidence for a physiological role of prostaglandins in oviposition by the hen. J Reprod Fertil 53:71

Hirshfield AN 1991 Development of follicles in the mammalian ovary. Int Rev Cytol 124:43

Hirshfield AN, Midgley ARJ 1978 The role of FSH in the selection of large ovarian follicles in the rat. Biol Reprod 19:606

Hoeck WG, Ramesha CS, Chang DJ, Fan N, Heller R 1993 Cytosolic phospholipase A_2 activity and gene expression are stimulated by tumor necrosis factor: dexamethasone blocks the induced synthesis. Proc Natl Acad Sci USA 90:4475

Honn KV, Bockman RS, Marnett LJ 1981 Prostaglandins and cancer: a review of tumor initiation through tumor metastasis. Prostaglandins 21:833

Hoodless PA, Haerry T, Abdollah S, Stapleton M, O'Connor MB, Attisano L and Wrana JL 1996 MADR1, a MAD-related protein that functions in BMP2 signaling pathways. Cell 85:489

Horton ES, Poyer NL 1976 Uterine luteolytic hormone: A physiological role for prostaglandin $F_{2\alpha}$. Physiol Rev 56:595

Hsueh AJW, Adashi EY, Jones PB, Welsh TH Jr 1984 Hormonal regulation of the differentiation of cultured ovarian granulosa cells. Endocrinol Rev 5:76

Hsueh AJW, Billing H, Tsafriri A 1994 Ovarian follicle atresia: a hormomally controlled apoptotic process. Endocrinol Rev 15:1

Huang NN, Wang DJ, Gonzalez F, Heppel LA 1991 Multiple signal transduction pathways lead to extracellular ATP-stimulated mitogenesis in mammalian cells: II. A pathway involving arachidonic acid release, prostaglandin synthesis, and cyclic AMP accumulation. J. Cell. Physiol. 146:483

Huarte J, Belin D, Bosco D, Sappino AP, Vassalli JD 1987 Plasminogen activator and mouse spermatozoa: urokinase synthesis in the male genital tract and binding of the enzyme to the sperm cell surface. J Cell Biol 104:1281

Hughes FMJ, Gorospe WC 1991 Biochemical identification of apoptosis (programmed cell death) in granulosa cells: evidence for a potential mechanism underlying follicular atresia. Endocrinology 129:2415

Hurwitz A, Payne DW, Packman JN, Andreani CL, Resnick CE, Hernandez ER, Adashi EY 1991 Cytokine-mediated regulation of ovarian function: interleukin-1 inhibits gonadotropin-induced androgen biosynthesis. Endocrinology 129:1250

Hutchinson LA, Findlay JK, De vos FL, Robertson DM 1987 The effects of bovine inhibin, transforming growth factor-β and bovine activin-A on granulosa cell differentiation. Biochem Biophys Res Commun 146:1405

Inagaki M, Wang Z, Carr BI 1994 Transforming growth factor beta 1 selectively increases gene expression of the serine/threonine kinase receptor 1 in human hepatoma cell lines.

Cell Struct Funct 19:305

Ireland JJ 1987 Control of follicular growth and development. J Reprod Ferti (suppl) 34: 39

Jackson JA, Tischkau SA, Zhang P, Bahr JM 1994 Plasminogen activator production by the granulosa layer is stimulated by factor (s) produced by the theca layer and inhibited by the luteinizing hormone surge in the chicken. Biol Reprod 50:812

Jackson JA, Zhang P, Bahr JM 1993 Plasminogen activator activity in preovulatory follicles during the ovulatory cycle of the chicken. Biol Reprod 49:1141

Jaffe BM, Behrman HR, Parker CW 1973 Radioimmunoassay measurement of PGE, A and F in human placenta. J Clin Invest 54:398

Johnson AL, Bridgham JT, Anthony RV 1997 Expression of avian urokinase and tissue-type plasminogen activator messenger ribonucleic acid during follicle development and atresia. Biol Reprod 56:581

Johnson AL, Bridgham JT, Wagner B 1996 Characterization of a chicken luternizing hormone receptor (cLH) complementary deoxyribonucleic acid, and expression of cLF-R messenger ribonucleic acid in the ovary Biol Reprod 55: 304

Johnson AL, Bridgham JT, Witty JP, Tilly JL 1996 Susceptibility of avian ovarian granulosa cells to apoptosis is dependent upon stage of follicle development and is related to endogenous levels of bcl-xlong gene expression. Endocrinology 137: 2059

Jones PBC, Welsh TH, Hsueh AJW 1982 Regulation of ovarian progestin production by epidermal growth factor in cultured rat granulosa cells. J Biol Chem 257:11268

Karakji EG, Tsang BK 1995a Tumor necrosis factor alpha inhibits rat granulosa cell plasminogen activator activity *in vitro* during follicular development. Biol Reprod 53:544

Karakji EG, Tsang BK 1995b Follicular stage-dependent regulation of rat granulosa plasminogen activator system by transforming growth factor-alpha *in vitro*. Biol Reprod 52:1050

Karakji EG, Tsang BK 1995c Regulation of rat granulosa cell plasminogen activator system: influence of interleukin-1β and follicular development. Biol Reprod 53:1302

Karakji EG, Tsang BK 1995d Growth hormone releasing factor and vasoactive intestinal peptide stimulate rat granulosa cell plasminogen activity *in vitro* during follicular development. Mol Cell Endocrinol 107:105

Kawano T, Mormoto K, Uemura Y 1970 Partial purification and properties of urokinase inhibitor from human placenta. J Biochem 67:333

Khan SA, Schmidt K, Hallin P, Pauli RDI, DE Geyter CH, Nieschlag E 1980 Human testis and cytosol and ovarian fluid contain high amount of interleukin-1-like factors. Mol Cell Endocrinol 58:221

Kim I-C, Schomberg DW 1989 The production of transforming growth factor - β activity by rat granulosa cell cultures. Endocrinology 124:1345

Kingsley DM 1994 The TGFβ superfamily: new members, new receptors and new genetic tests of function in different organisms. Genes Dev 8:133

Kirchheimer JC, Wojta J, Christ G, Binde B 1987 Proliferation of a human epidermal cell line stimulated by urokinase. FASEB J1:125

Kirkwood AP, O'Banion MK, Miano JM, Vlasic N, Bhatia UG, Young DA, Stemerman MB 1994 Induction of cyclooxygenase-2 in rat vascular smooth muscle cells *in vitro* and *in Vivo*. J Biol Chem 269:8504

Kirshino J, Tohkin M, Arta H 1992 Proliferative effect of phospholipase A2 in rat chondrocyte via its specific binding sites. Biochim Biophys Acta 1125:210

Kishino J 1992 Proliferative effect of phospholipase A2 in rat chondrocyte via its specific binding sites. Biochem Biophys Res Commun 186: 1025

Knecht M, Feng P, Catt KJ 1987 Bifunctional role of transforming growth factor beta during granulosa cell development. Endocrinology 120:1243

Komoriya A, Hotsch M, Meyers C, Smith M, Kanety H, Schlessinger J 1984 Biologically active synthetic fragments of epidermal growth factor: localization of major receptor-binding region. Proc Natl Acad Sci USA 81:1351

Kol S, Ruutiainen-Altman K, Ben-Shlomo I, Payne DW, Ando M, Adashi EY 1997a The rat ovarian phospholipase A_2 system: gene expression, cellular localization, activity characterization, and interleukin-1 dependence. Endocrinology 138: 322

Kol S, Ben-Shlomo I, Ando M, Payne DW, Adashi EY 1997b Interleukin-1 beta stimulates ovarian phospholipase A_2 (PLA_2) expression and activity: up-regulation of both secretory and cytosolic PLA_2. Endocrinology 138: 314

Kramer RM, Roberts EF, Manetta J and Putnam JE 1991 The Ca^{2+}-sensitive cytosolic phospholipase A_2 in human platelets. J Biol Chem 268:26796

Kudlow J, Kobrin MS, Purchio AF, Twardzik DR, Hernandes K, Asa SL, Adashi EY 1987 Ovarian transforming growth factor-α gene expression: immunohistochemical localization to the theca interstitial cells. Endocrinology 121:1577

Kujubu DA, Reddy ST, Fletcher BS, Herschman HR 1993 Expression of the protein product of the prostaglandin synthase-2/TIS10 gene in mitogen-stimulated Swiss 3T3 cells. J Biol Chem 268:5425

Kujubu DA, Fletcher BS, Varnum BC, Lim RW, Herschman HR 1991 TIS10, a phorbol ester tumor promoter-inducible mRNA from Swiss 3T3 cells, encodes a novel prostaglandin synthase/cyclooxygenase homologue. J Biol Chem 266:12866

Kuo ML & Yang NC 1995 Reversion of v-H-ras-transformed NIH 3T3 cells by apigenin through inhibiting mitogen activated protein kinase and its downstream oncogenes. Biochem. Biophys Res Commun 212:767

Labarca C, Paigen K 1980 A simple, rapid and sensitive DNA assay procedure. Anal Biochem 102:344

Lafrance M, Zhou L, Tsang BK 1993b Interactions of transforming growth factor -α and -β and luteinizing hormone in the regulation of plasminogen activator activity in avian granulosa cells during follicular development. Endocrinology 133:702

Lafrance M, Croze F, Tsang BK 1993a Influence of growth factors on the plasminogen activator activity of avian granulosa cells from follicles at different maturational stages of preovulatory development. J Mol Endocrinol 11:291

Lagna G, Hata A, Hemmati-Brivanlou A, Massauge J 1996 Partnership between DEPC4 and SMAD protein in TGFβ signalling pathways. Nature 383:832

Lands WEM 1979 The biosynthesis and metabolism of prostaglandins. Rev Physiol 41:633

LaPolt PS, Tilly J, Aihaia T, Nishimari K, Hsueh AJW 1992 Gonadotropin-induced up- and down-regulation of ovarian follicle-stimulating hormone (FSH) receptor gene expression in immature rats: effect of pregnant mare's serum gonadotropin, human chorionic gonadotropin, and recombinant FSH. Endocrinology 130:1289.

Law As, Burt DW, Armstrong DG 1995 Expression of transforming growth factor beta mRNA in chicken ovarian follicular tissue. Gen Compar Endocrinol 98:227

Lax L, Johnson A, Howk R, Sap J, Bellot F, Winkler M, Uurich A, Vennstrom B,

Schlessinger J, Givol D 1988 Chicken epidermal growth factor (EGF) receptor: DNA cloning, expression in mouse cells, and differential binding of EGF and transforming growth factor alpha. Mol Cell Biol 8: 1970

Leal JA, May JV, Keel BA 1990 Human alpha fetoprotein enhances epidermal growth factor proliferative activity upon porcine granulosa cells in monolayer culture. Endocrinology 126:669

Ledwith BJ, Pauley CJ, Wagner LK, Rokos CL, Alberts DW, Manam S 1997 Induction of COX II expression by peroxisome proliferators and non-tetradecanoylphorbol 12,13-myristate-type tumor promoters in immortalized mouse liver cells. J Biol Chem 272: 3707

LeMaire WJ, Yang NST, Behrman HR and Marsh JM. 1973 Preovulatory changes in the concentration of prostaglandins in rabbit graafian follicles. Prostaglandins 3:367

Leslie ND, Kesser CA, Bell SM, Degen JL 1990 The chicken urokinase-type plasminogen activator gene. J Biol Chem 265:1339

Levin EG, Loskutoff DJ 1982 Cultured bovine endothelial cells produce both urokinase and tissue-type plasminogen activator. J Cell Biol 94:631

Lewis GS, Jenkins PE, Fogwell RL, Inskeep EK 1978 Concentration of prostaglandin E_2 and $F_{2\alpha}$ and their relationship to luteal function in early pregnant ewes. J Anim Sci 47:1314

Li J, Li M, Lafrance M, Tsang BK 1994 Avian granulosa cell prostaglandin secretion is regulated by transforming growth factor α and β and does not control plasminogen activator activity during follicular development. Biol Reprod 51:787

Li J, Li M, Tsang BK 1997 Cytosolic phospholipase A_2 in hen granulosa cell is regulated by transforming growth factors during follicular development. Biol Reprod 57:929-935.

Li J, Simmons DL, Tsang BK 1996 Regulation of hen granulosa cell prostaglandin production by transforming growth factors during follicular development: involvement of cyclooxygenase II. Endocrinology 137:2522

Li J, Tsang BK 1995 Prostaglandins mediate the stimulation of deoxyribonucleic acid synthesis by transforming growth factor α in hen granulosa cells during ovarian follicular development. Biol Reprod 52:1050

Lin JK, Chou CK 1992 In vitro apoptosis in the human hepatoma cell line induced by transforming growth factor beta 1. Cancer Res 52: 385

Lin L-L, Wartmann M, Lin AY Knopf JL, Seth A and Davis RJ 1993 $cPLA_2$ is phosphorylated and activated by MAP kinase. Cell 72:269

Lipner H 1988 Mechanism of Mammalian Ovulation. In: The Physiology of Reproduction, vol 1. Knobil E, Neill JD (ed.). Raven Press, New York, pp447-488.

Liu F, Hata A, Baker J, Doody J, Carcamo J, Harland R, Massaque J 1996 A human MAD protein acting as a BMP-regulated transciptional activator. Nature 381, 620

Liu X., Peng XR, Ny T 1991 Tissue-specific and time coordinated hormone regulation of plasminogen activator inhibitor type 1 and tissue-type plasminogen activator in the rat ovary during gonadotropin-induced ovulation. Eur J Biochem 195:549

Liu Y, Stephen C 1988 Indomethacin inhibits hCG and GnRH agonist-induced secretion of plasminogen activator by granulosa and theca interstitial cells of hypophysectomized rats. Sci Sinica 31:807

Liu YX, Cajander SB, Ny T, Kristensen P, Hsueh AJY 1987 Gonadotropin regulation of tissue-type and urokinase-type plasminogen activators in rat granulosa and theca-interstitial cells during the periovulatory period. Mol Cell Endocrinol 54:221

Lobb DK, Kolorin MS, Kudlow JE, Dorrington JH 1989 Transforming growth factor-α in the adult bovine ovary: identification in growing ovarian follicle. Biol Reprod 40:1087

Loskutoff DJ, Sawdey M, M imuro J 1988 Type 1 plasminogen activator inhibitor. In: Progressing Haemostasis and Thrombosis. B.S. Coller, ed. Saunders, Philadelphia, pp 87

Magoffin DA, Erickson GF 1982 Primary culture of differentiating ovarian androgen-producing cells in defined medium. J Biol Chem 257:4507

Magoffin DA, Gancedo B, Erickson GF 1989 Transforming growth factor - β promotes differentiation of ovarian thecal-interstitial cells but inhibits androgen production. Endocrinology 125: 1951

Mallat A, Fourassier L, Preaux AM, Mavier P, Lotersztajn S 1995 Antiproliferative effects of ET-1 in human liver Ito cells: an ETB- and a cylic AMP-mediated pathway. J Cardiovasc Pharmacol 26 Suppl 3:S132

Marcinkiewicz JL, Krishna A, Cheung CM, Terranova PF 1994 Oocytic tumor necrosis factor alpha: Localization in the neonatal ovary and throughout follicular development in the adult rat. Biol Reprod 50:1251

Marquant H, Hunkapiller MW, Hood LE, Todaro GJ 1984 Rat transforming growth

factor type 1: structure and relation to epidermal growth factor. Science 223:1079

Marza ME, Marza EV 1953 The formation of hen's egg. I-IV. Q. J Microsc. Science 78: 134

Massague J 1996 TGFβ signaling; receptor, transducters and Mad protein. Cell 85:357

Massague J , Attisano L and Wrana JL 1996 The TGFβ family and its composite receptors. Trends Cell Biol 4:172

Mathews LS, Vale WW 1991 Expression cloning of an activin receptor, a predicted transmembrane serine kinase. Cell 65:973

Mathieu C, Jozan S, Mazars P, Come MG, Moisand A, Valette A 1995 Density-dependent induction of apoptosis by transforming growth factor beta 1 in human ovarian carcinoma cell line. Exp Cell Res 216:13

May JV, Schomberg DW 1989 The potential relevance of epidermal growth factor and transforming growth factor alpha to ovarian physiology. Sem in Reprod Endocrinol 7:1

Mckenzie FR, Pouyssegur J 1996 cAMP-mediated growth inhibition in fibroblasts is not mediated via mitogen-activated protein (MAP) kinase (ERK) inhibition. cAMP-dependent protein kinase induced a temporal shift in growth factor-stimulated MAP kinases. J Biol Chem 271:13476

Miro F, Smyth CD, Hillier SG 1991 Development-related effects of recombinant activin on steroid synthesis in rat granulosa cells. Endocrinology 129:3388

Mohammadi M 1993 Aggregation-induced activation of the epidermal growth factor receptor protein tyrosine kinase. Biochem. 32:8193

Mondschein JS, Schomberg DW 1981 Growth factors modulate gonadotropin receptor induction in granulosa cell cultures. Science 211:1179

Moolenaar WH, Aerts RJ, Tertoolen LCJ, Deleat SW 1986 The epidermal growth factor-induced calcium signal in A431 cells. J Biol Chem 261:279

Moran MF, Koch CA, Anderson D, Ellis C, England L, Martin GS, Pawson T 1990 Src homology region 2 domains direct protein-protein interactions in signal transduction. Proc Natl Acad Sci USA 87:8622

Morrison P, Saltiel AR, Rosner MR 1996 Role of mitogen-activated protein kinase in regulation of the epidermal growth factor receptor by protein kinase C. J Biol Chem

271:12891

Mulheron GW, Schomberg DW 1990 Rat granulosa cells express transforming growth factor β type 2 messenger ribonucleic acid which is regulated by follicle-stimulating hormone *in vitro*. Endocrinology 126:1777

Murdock WJ, Peterson TA, Van Kirk EA, Vincent DL, Inskeep EK 1986 Interactive roles of progesterone, prostaglandins and collagenase in the ovulatory mechanism of the ewe. Biol Reprod 35:187

Nagamine Y, Pearson D, Altus MS, Reich E 1984 cDNA and gene nucleotide sequence of porcine plasminogen activator. Nucleic Acids Res 12:9525

Nahas N, Waterman WH, Sha'afy R 1996 Granulocyte-macrophage colony-stimulating factor (GM-CSF) promotes phosphorylation and an increase in the activity of cytosolic phospholipase A_2 in human neutrophils. J Biochem 313:503

Nakamura T, Lin L-L, Kharbanda S, Knopf J, Kufe D 1992 Macrophage colony stimulating factor activates phosphatidycholine hydrolysis by cytoplasmic phospholipase A_2. J EMBO 11:4917

Newfeld SJ, Chartoff EH, Graff JM, Melton DA, Gelbart WM 1996 Mothers against dpp encodes a conserved cytoplasmic protein required in DPP/TGFβ-responsive cells. Development 122: 2099

Niehrs C 1996 MAD connection to the nucleus. Nature 381: 561

Nielsen LS, Kellerman GM, Behrendt N, Picone R, Dano K, Blasi F 1988 A 55,000-60,000 Mr receptor protein for urokinase-type plasminogen activator: identification in human tumor cell lines and partial purification. J Biol Chem 263:2358

Niswender GD, Nett TM 1988 The corpus luteum and its control. In: The Physiology of Reproduction, vol 1. Knobil E, Neill JD (ed.). Raven Press, New York, pp489

Nolan RD, Danilowicz RM, Elin TE 1988 Role of arachidonic acid metabolism in the mitogenic response of BALB/c3T3 fibroblasts to epidermal growth factor. Mol Pharmacol 33:650

Nomura K, Fujita H, Arita H 1994 Gene expression of pancreatic-type phospholipase-A_2 in rat ovaries:stimulatory action on progesterone release. Endocrinology 135: 603

Ny T, Bjersing L, Hsueh AJW, Loskutoff DJ 1985 Cultured granulosa cells produce two plasminogen activators and an anti-activator, each regulated differently by gonadotropins.

Endocrinology 116:1666

Ny T, Elgh F, Lund B 1984 The structure of the human tissue-type plasminogen activator gene: correlation in intron and exon structures to functional and structural domain. Proc Natl Acad Sci USA 81:5355.

O'Banion MK, Sadowski HB, Winn V, Young DA 1991 A serum- and glucocorticoid-regulated 4-kilobase mRNA encodes a cyclooxygenase-related protein. J Biol Chem 266:23261

O'Connell ML, Canapari R, Strickland S 1987 Hormonal regulation of tissue plasminogen activator secretion and mRNA levels in rat granulosa cells. J Biol Chem 262:2339

Onagbesan OM, Gullick W, Woolveridge I, Peddie MJ 1994 Immunohistochemical localization of epidermal growth factor receptor, epidermal-growth-factor-like and transforming-growth-factor-alpha-like peptide in chicken ovarian follicles. J Reprod Fertil 102:47

Ossowski L, Biegel D, Reich E 1976 Mammary plasminogen activator: correlation with involution, hormonal modulation and comparison between normal and neoplastic tissue. Cell 16:929

Ossowski L, Reich E 1983 Antibodies to plasminogen activator inhibit human tumor metastasis. Cell 35:611

Padgett RW, St. Johnston RD, Gelbart W 1987 A transcript from a Drosophila pattern predicts a protein homologous to the transforming growth factor-beta family. Nature 325:81

Peddie MJ, Onagbesan OM, Williams J 1994 Chicken granulosa cell proliferation and progesterone production in culture: effects of EGF and theca secretions. Gen Comp Endocrinol 94:341-356.

Peng XR, Hsueh AJW, Ny T 1993 Transient and cell specific expression of tissue-type plasminogen activator and plasminogen activator inhibitor type 1 results in a controlled and directed proteolysis during gonadotropin-induced ovulation. Eur J Biochem 214:147

Pennica D, Holmes WE, Kohr WJ, Harkins RN, Vehar GA 1983 Cloning and expression of human tissue-type plasminogen activator cDNA in *E coli*. Nature 301:214

Piquette GN, LaPolt PS, Oikawa M, Hsueh AJW 1991 Regulation of LH receptor messenger ribonucleic acid levels by gonadotropins, growth factors and GnRH in cultured rat granulosa cells. Endocrinology 128:2449

Plow EF, Miles LA 1990 Plasminogen receptors in the mediation of pericellular proteolysis. Cell Differ Dev. 32:293

Politis I, Wang L, Turner JD, Tsang BK 1990 Changes in tissue-type plasminogen activator-like and plasminogen activator inhibitor activities in granulosa and theca layers during ovarian follicular development in domestic hen. Biol Reprod 42:747

Propping D, Zaneveld LJD, Tauber PF, Schumacher GFB 1978 Purification of plasminogen activators from human seminal plasma. Biochem J 171:435

Ptosed JD, Scanter P, Haired J, Ruegg M, Kruithof EKO, Buchmann F 1982 Isolation from human plasma of a plasminogen activator identical to urinary high molecular weight urokinase. J Clin Invest 70:1320.

Quirk SM, Cowan RG, Joshi SG, Henrikson KP 1995 Fas antigen-mediated apoptosis in human granulosa/luteal cells. Biol Reprod 52:279

Ranby M, Bergsdorf N, Pohl G, Wallen P 1982 Isolation of two variants of native one-chain tissue plasminogen activator. FEBS Lett 146:289

Refsnes M, Thoresen GH, Dajani OF, Christoffersen T 1994 Stimulation of hepatocyte DNA synthesis by prostaglandin E_2 and prostaglandin $F_{2\alpha}$: additivity with the effect of norepinephrine, and synergism with epidermal growth factor. J Cell Physiol 159:35

Riccio A, Grimaldi G, Verde P, Sebastio G, Boast S, Blasi F 1985 The human urokinase-plasminogen activator gene and its promoter. Nucleic Acids Res 13:2759

Richards JS 1980 Maturation of ovarian follicles: actions and interactions of pituitary and ovarian hormones on follicular cell differentiation. Physiol Rev 60:51.

Richards JS, Bogovich K 1982 Effect of human chorionic gonadotropin and progesterone on follicular development in the immature rat. Endocrinology 111:1429

Richards JS, Midgley AR Jr 1976 Protein hormone action: a key to understanding ovarian follicular and luteal cell development. Biol Reprod 14:82.

Rickles RJ, Darrow AL, Strickland S 1988 Molecular cloning of complementary DNA to mouse tissue-plasminogen activator mRNA and its expression during FQ teratocarcinoma cell differentiation. J Biol Chem 263:1563

Riech R, Miskin R 1985 Tsafriri A Follicular plasminogen activator: involvement in ovulation. Endocrinology 116:516

Rijken DC, Wijngaards G, Welbergen J 1981 Immunological characterization of plasminogen activators in human tissues and body fluids. J Lab Clin Med 97:477

Ritzhaupt LK, Bahr JM 1987 Adecrease in FSH receptor of granulosa cells during follicular maturation in the domestic hen J Endocrinol 115: 303

Robbins KC, Summaria L, Hsieh B, Shah J 1967 The peptide chains of human plasmin. Mechanism of activation of human plasminogen to plasmin. J Biol Chem 241:2333

Roby KF, Terranova PF 1988 Tumor necrosis factor alpha alters follicular steroidogenesis in vitro. Endocrinology 123:2952.

Roby KF, Terranova PF 1989 Localization of tumor necrosis factor (TNF) in rat and bovine ovary using immunocytochemistry and cell blot: evidence for granulosa cell production. In: Growth Factors and the Ovary. Hirshfield A (ed). Plenum Press, New York, p273

Rosen LB, and Greenberg ME 1996 Stimulation of growth factor receptor signal transduction by activation of voltage-sensitive calcium channels. Proc Natl Acad Sci USA 93:1113

Rouzer CA, Ford-Hutchinson AW, Morton HE, Gillard JW 1990 MK886, a potent and specific leukotriene biosynthesis inhibitor blocks and reverses the membrane association of 5-lipoxygenase in ionophore-challenged leukocytes. J Biol Chem 265:136

Roy SK, Greenwald GS 1990 Immunohistochemical localization of epidermal growth factor-like activity in the hamster ovary with polyclonal antibody. Endocrinology 126:1309

Rzasa J 1978 Effects of arginine vasotocin and prostaglandin E_1 on the hen uterus. Prostaglandins 16:357

Sakata T, Nakamura E, Tsuruta Y, Tamaki M, Teraoka H, Tojo H, Ono T, Okamoto M 1989 Presence of pancreatic-type phospholipase A2 from porcine pancreas. Biochim Biophys Acta 159:118

Saksela O 1985 Plasminogen activation and regulation of pericellular proteolysis. Biochim Biophys Acta 823:35

Saksela O, Moscatelli D, Sommer A, Rifkin DB 1988 Endothelial cell-derived heparan sulfate binds basic fibroblast growth factor and protect it from proteolytic degradation. J Cell Biol 107:743.

Salustri A, Ulisse S, Yanagishita M, Hascall VC 1990 Hyaluronic acid synthesis by mural granulosa cells and cumulus cells *in vitro* is selectively stimulated by a factor produced by

oocytes and transforming growth factor-beta. J Biol Chem 265:19517

Sappino AP, Huarte J, Belin D, Vassalli JD 1989 Plasminogen activators in tissue remodelling and cell invasion: mRNA localization in mouse ovaries and implanting embryos. J Cell Biol 109:2471

Satoh H, Takaoka H, Makinoda S, Moriya S, Ichinoe K 1985 On the effect of an oocyte factor on the formation of cumulus oophorus in human antral follicles. Nippon Sanka Fujinka Gakkai Zasshi 37:1185

Savage C, Das P, Finelli A, Townsend S, Sun C, Baird S, Padgett R 1996 The C. Elegans sma-2, sma-3 and sma-4 genes define a novel conserved family of TGFβ pathway components. Proc Natl Acad Sci USA 93:790

Schievella AR, Regier MK, Smith WL, and Lin L-L 1995 Calcium-mediated translocation of cytosolic phospholipase A_2. J Biol Chem 270:30749

Schlessinger J, Ullrich A 1992 Growth factor signaling by receptor tyrosine kinase. Neuron 9:383

Schomberg DW, May JV, Mondschein JS 1983 Interactions between hormones and growth factors in the regulation of granulosa cell differentiation *in vitro*. J Steroid Biochem 19:291

Schuler GD, Cole MD 1988 GM-CSF and oncogene mRNA stabilities are independently regulated in *trans* in a mouse monocytic tumor. Cell 55:1115

Segaloff DL, Wang H, Richards JS 1990 Hormonal regulation LH/chorionic gonadotropin receptor mRNA in rat ovarian cells during follicular development and luteinization. Mol Endocrinology 4:1856

Seilhamer JJ, Pruzanski W, Vadas P, Plant S, Miller JA, Kloss J, Johnson LK 1989 Cloning and recombinant expression of phospholipase A_2 present in rheumatoid arthritic synovial fluid. J Biol Chem 264:5335

Sekelsky JJ, Newfeld SJ, Raftery LA, Chartoff EH and Gelbart WM 1995 Genetic characterization and cloning of Mothers against dpp, a gene required for decapentaplegic function in Drosophila melanogaster. Genetics 139:1347

Shaw G, Kamen R 1986 A conserved AU sequence from the 3' untranslated region of GM-CSF mRNA mediates selective mRNA degradation. Cell 46:4888

Shimada K, Asai I 1979 Effects of prostaglandin $F_2\alpha$ and indomethacin on uterine contraction in hens. Biol Reprod 21:523

Shimada H, Okamura H, Noda Y, Suzuki A, Jojo S, Takada AJ 1983 Plasminogen activator in rat ovary during the ovulatory process, independence of prostaglandin mediation. Endocrinology 97:201

Shull MM, Doetschman T 1994 Transforming growth factor beta 1 in reproduction and development. Mol Reprod Dev 39: 239

Simon C, Frances A, Piquette G, Polan ML 1994 Immunohistochemical localization of the interleukin-1-system in the mouse ovary during follicular growth, ovulation and luteinization. Biol Reprod 50:449

Simmons DL, Levy DB, Yannoni Y, Erikson RL 1989 Identification of a phorbol ester-repressible v-src-inducible gene. Proc Natl Acad Sci USA 86:1178

Simmons KR, Caffrey JO, Phillips JL, Abel JH, Niswender GD 1976 A simple method for preparing suspension of luteal cells. Proc Soc Exp Biol Med 148:123

Sirois J, Richards JS 1992 Purification and characterization of a novel, distinct isoform of prostaglandin endoperoxide synthase induced by human chorionic gonadotropin in granulosa cells of rat preovulatory follicles. J Biol Chem 267:6382

Sirois J 1994 Induction of prostaglandin endoperoxide synthase-2 by human chorionic gonadotropin bovine preovulatory follicles *in vivo*. Endocrinology 135:841

Sirois J, Simmons DL, Richards JS 1992 Hormonal regulation of messenger ribonucleic acid encoding a novel isoform of prostaglandin endoperoxide H synthase in rat preovulatory follicles. J Biol Chem 267:11586

Skinner MK, Dorrington JH 1984 Control of fibronectin synthesis by rat granulosa cell in culture. Endocrinology 115:2029.

Skinner MK, Coffey RJ 1988 Regulation of ovarian cell growth through the local production of transforming growth factor - α by theca cells. Endocrinology 123:2632

Skinner MK, Keski-Oja J, Osteen K, Moses HL 1987 Ovarian thecal cells produce transforming growth factor β which can regulate granulosa cell growth. Endocrinology 121786

Skinner MK, McKeracher HL, Dorrington JH 1985 Fibronectin as a marker of granulosa cell differentiation. Endocrinology 117:886.

Smith WC, Mckendry R, Ribisi S, Harland RM 1995 A nodal-related gene defines a

physical and functional domain within the Spermann organizer. Cell 82: 36-47

Soboloff J, Désilets M, Tsang BK 1994 The influence of the muscarinic agonist carbachol on intracellular Ca^{2+} in chicken granulosa cells: I.dependence on follicular maturation. In: Proceedings of the 41st Annual Meeting of the Society for Gynaecologic Investigation,Chicago, IL. Abstract # P297.

Stoppelli MP, Corti A, Soffientini A, Cassani G, Blasi F, Assoian RK 1985 Differentiation-enhanced binding of the amino-terminal fragment of human urokinase plasminogen activator to a specific receptor on U937 monocytes. Proc Natl Acad Sci USA 82:4939.

Strickland S, Beers WH 1976 Studies on the role of plasminogen activator in ovulation. J Biol Chem 251:5694

Strickland S, Reich E 1976 Plasminogen activator in early embryogenesis: enzyme production by trophoblast and partial endoderm. Cell 9:231

Stump DC, Lijnen HR, Collen D 1986 Purification and characterization of a novel low molecular weight form of single-chain urokinase-type plasminogen activator. J Biol Chem 261: 17120

Sun FF, Chapman JP, McGuire JC 1977 Metabolism of prostaglandin endoperoxides in animal tissues. Prostaglandins 14:1055

Tahar M, Tasaka K, Musumoto N, Adachi K, Adachi H, Ikebuchi Y, Kurachi H, Miyake A 1995 Expression of messenger ribonucleic acid for epidermal growth factor (EGF), transforming growth factor-alpha (TGF alpha), and EGF receptor in human amnion cells: possible role of TGF alpha in prostaglandin E2 synthesis and cell proliferation. J Clin Endocrinol Metabol 80:138

Takahide M 1990 Immuno-endocrinology of cyclic ovarian function. Am J Reprod Immunol 23:80

Takakura K, Taii S, Fukuoka M, Yasuda Y, Tagaya J, Yodoi J, Mori T 1989 Interleukin-1 receptor/p55(tac) inducing activity in porcine follicular fluid. Endocrinology 125:618

Takehara Y, Dharmarajan AM, Kaufman G, Wallach EE 1994 Effect of interleukin-1β on ovulation in the *in vitro* perfused rabbit ovary. Endocrinology 134:1788

Thomsen GH 1996 Xenopus mothers against decapentaplegic is an embryonic ventralizating agent that acts downsteam of the BMP2/ 4 receptor. Development 122:2359

Tigyi G, Dyer DL, Miledi R 1994 Lysophosphatidic acid possesses dual action in cell

proliferation. Cell Biol 91:1908

Tilly JL, Billing H, Kowalski H, Hsueh AJW 1992a Epidermal growth factor and basic fibroblast growth factor suppress the spontaneous onset of apoptosis in cultured rat ovarian granulosa cells and follicles by a tyrosine kinase-dependent mechanism. Mol Endocrinol 92:1942

Tilly JL, Johnson AL 1990a Modulation of hen granulosa cell steroidogenesis and plasminogen activator activity by transforming growth factor alpha. Growth Factors 3:247

Tilly JL, Johnson AL 1990b Effect of several growth factors on plasminogen activator activity in granulosa and theca cells of the domestic hen. Poultry Sci 69:292

Tilly JL, Johnson AL 1987 Presence and hormonal control of plasminogen activator in granulosa cells of the domestic hen. Biol Reprod 37:1156

Tilly JL, Kowalski KI, Li I, Levorse JM, Johnson AL 1992b Plasminogen activator activity and thymidine incorporation in avian granulosa cell during follicular development and the preovulatory period. Biol Reprod 46:195

Tilly JL, Kowalski KI, Levorse JM, Johnson AL, Hsueh AJW 1991 Involvement of apoptosis in ovarian follicular atresia and postovulatory regression. Endocrinology 129:2799

Tischkau SA, Jackson JA, Bahr JM 1993 The germinal disc region is the proliferative center of the of the follicle. Biol Reprod 48 (suppl 1):152 (abstract 374)

Tischkau SA, Jackson JA, Finnigan-Bunick C, Bahr JA 1996 Granulosa layer: primary site of regulation of plasminogen activator messenger ribonucleic acid by luteinizing hormone in the avian ovary. Biol Reprod 55:75

Todaro GJ, Fryling C, DeLarco JE 1980 Transforming growth factors produced by certain human tumor cells: polypeptides that interact with epidermal growth factor receptors. Proc Nat Acad Sci USA 77:5258

Tohkin M, Kishino J, Ishizaki J, Arita H 1993 Pancreatic-type phospholipase A2 stimulates prostaglandin synthesis in mouse osteolastic cells (MC3T3) via a specific binding site. J Biol Chem 268:2865

Tojo H, Ono T, Kuramistu S, Okamoto M 1988 A phospholipase A2 in the supernatant fraction of rat spleen: its similarity to rat pancreatic phospholipase A2. J Biol Chem 263:5724

Too CKL, Bryant-Greenwood GD, Greenwood FC 1984 Relaxin increases the release of plasminogen activator, collagenase, and proteoglycanase from rat granulosa cell *in vitro*.

Endocrinology 115:1043

Tsafriri A, Bicsak TA, Cajander SB, NY T, Hsueh AJW 1989 Suppression of ovulation rate by antibodies to tissue-type plasminogen activator and a-antiplasmin. Endocrinology 124:415

Tsafriri A, Hoch Y, Lindner HR 1973 Ovulation rate and serum LH levels in rats treated with indomethacin or prostaglandin E_2. Prostaglandins 3:461

Tsang BK, Ainsworth L, Downey BR, Armstrong DT 1979 Preovulatory changes in cyclic AMP and prostaglandin concentrations in follicular fluid of gilts. Prostaglandins 17:141

Tsang BK, Arodi J, Li M, Ainsworth L, Srikandakumar A, Downey BR 1988 Gonadotropic regulation of prostaglandin production by ovarian follicular cells of pig. Biol Reprod 42:168

Uilenbroek JTJ, Richards JS 1979 Ovarian follicular development during the rat estrous cycle: gonadotropin receptors and follicular responsiveness. Biol Reprod 20:1159.

Unemorl EN, Ehsani N, Wang M, Lee S, Mcguire J, Amento EP 1994 Interleukin-1 and transforming growth factor-α: synergistic stimulation of metalloproteinases, PGE_2, and proliferation in human fibroblasts. Exp Cell Res 210:166

Vadas P, Stefanski E, Pruzanski W 1985 Characterization of extracellular phospholipase A_2 in rheumatoid synovial fluid. Life Sci 36:579

Van Mourik, JA, Lawrence DA, Loskutoff DJ 1984 Purification of an inhibitor of plasminogen activator (antiactivator) synthesized by endothelial cells. J Biol Chem 259:14914

Van Obberghen-Schilling E, Roche NS, Flander KC, Sporn MB, Roberts AB 1988 Transforming growth factor beta 1 positively regulates its own expression in normal and transformed cell. J Biol Chem 263: 7741

Van Zonneveld AJ, Veerman H, Pannekoek H 1986 On the interaction of the finger and the kringle-2 domain of tissue-type plasminogen activator with fibrin. Inhibition of kringle-2 binding to fibrin by epsilon-amino caproic acid. J Biol Chem 261:14214

Vanderhyden BC, Cohen JN, Morley P 1993 Mouse oocytes regulate granulosa cell steroidogenesis. Endocrinology 133:423

Vanderhyden BC, Tonary AM 1995 Differential regulation of progesterone and estradiol production by mouse cumulus and mural granulosa cells by a factor (s) secreted by the oocyte. Biol Reprod 53: 1243

Vassalli, J-D, Baccino D and Belin D 1985 A cellular binding site for the M_r 55,000 form

of the human plasminogen activator, urokinase. J Cell Biol 100:86

Verde P, Stoppelli MP, Gallefi P, DiNocera P, Blasi F 1984 Identification of the primary sequence of an unspliced human urokinase poly(A)$^+$ RNA. Proc Natl Acad Sci USA 81:4727

Vlodavsky I, Brown KD, Gospodarowicz D 1978 A comparison of the binding of epidermal growth factor to cultured granulosa and luteal cells. J Biol Chem 253:3744

Vlodavsky I, Folkman J, Sullivan R, Fridman R, Ishai-Michaeli R 1987 Endothelial cell-derived basic fibroblast growth factor: synthesis and deposition into subendothelial extra-cellular matrix. Proc Natl Acad Sci USA 84:2292

Wang LJ, Branstrom M, Robertson SA, Norman RJ 1992 Tumor necrosis factor alpha in the human ovary: presence in follicular fluid and effect on cell proliferation and prostaglandins production. Fertil Steril 58:934

Wang L, Croze F, Morley P, Tsang BK 1993 Granulosa-theca cell interaction in the regulation of plasminogen activator activity during ovarian follicular development in the hen. Biol Reprod 49: 924-932

Wechsung E, Houvenaghel A 1980 Influence of arachidonic acid and indomethacin on uterine and vaginal motility in the domestic hen. Poult Sci 59:2803

Wiersdroff V, Lecuit T, Cohen SM, Mlodzik M 1996 Mad acts downstream of dpp receptors revealing a differential requirement for dpp signaling in initiation and of morphogenesis in the Drosophila eye. Development 122: 2153

Wilson PA, Hemmati-Brivanlou A 1995 Induction of epidermis and inhibition of neural fate by BMP-4. Nature 376:331

Wohlwend A, Belin D, Vassalli JD 1987 Plasminogen activator-specific inhibitors in mouse macrophages: *in vivo* and *in vitro* modulation of their synthesis and secretion. J Immunol 139:1278.

Wong WY, Richards JS 1991 Evidence for two antigenically distinct molecular weight variants of prostaglandin H synthase in the rat ovary. Mol Endocrinol 5:1269

Wong WYL, Richards JS 1992 Induction of prostaglandin H synthase in rat preovulatory follicles by gonadotropin-releasing hormone. Endocrinology 130: 3512-

Wong WY, DeWitt DL, Smith WL, Richards JS 1989 Rapid induction of prostaglandin endoperoxide synthase in rat preovulatory follicles by luteinizing hormone and cAMP is blocked by inhibitors of transcription and translation. Mol Endocrinol 3:1714

Wrana JL, Attisano L, Wieser R, Ventura F, Massague J 1994 Mechanism of activation of the TGFβ receptor. Nature 370:341

Xie W, Chipman JG, Robertson DL, Erikson RL, Simmons DL 1991 Expression of a mitogen-responsive gene encoding prostaglandin synthase is regulated by mRNA splicing. Proc Natl Acad Sci USA 88:2692

Ye RD, Wun TC, Sadler JE 1987 DNA cloning and expression in *Escherichia coli* of plasminogen activator inhibitor from human placenta. J Biol Chem 262:3718

Yoshimura Y, Jinno M, Oda T, Shiokawa S, Yoshinaga A, Hanyu I, Akiba M, Nakamura Y 1994 Prolactin inhibits ovulation by reducing ovarian plasmin generation. Biol Reprod 50:1223

Yu K, Bayona W, Kallen CB, Harding HP, Ravera CP, McMahon G, Brown M, Lazar MA 1995 Differential activation of peroxisome proliferation-activated receptors by eicosanoids. J. Biol. Chem. 270: 23975

Yumoto N, Watanabe Y, Watanabe K, Watanabe Y, Hayaishi O 1986a Solubilization and characterization of prostaglandin E2 binding protein from porcine cerebral cortex. J Neurochem 46:125

Yumoto N, Hatanabe M, Watanabe K, Watanabe Y, Hayaishi O 1986b Involvement of GTP-regulatory protein in brain prostaglandin E2 receptor and separation of the two components. Biochem Biophys Res Commun 135:282

Zeleznik AJ, Hillier SG 1984 The role of gonadotropins in the selection of preovulatory follicle. Clin Obstet Gynecol 27:927

Zeleznik AJ, Ihrig LL, Bassett SG 1989 Developmental expression of Ca^{++}/Mg^{++}-dependent endonuclease activity in rat granulosa and luteal cells. Endocrinology 125:2218

Zeleznik AJ, Midgley AR Jr, Reichert LE Jr 1974 Granulosa cell maturation in the rat: increased binding of human chorionic gonadotropin following treatment with follicle-stimulating hormone *in vivo*. Endocrinology 95:818

Zhang Y, Feng X-H, Wu R-Y, Derynck R 1996 Receptor-associated Mad homologues synergize as effectors of the TGFβ response. Nature 383:168.

Zhang Z, Findlay JK, Carson RS, Herrington ACH, Burger HG 1988 Transforming growth factor beta enhances basal and FSH-stimulated inhibin production by rat granulosa cells *in vitro*. Mol Cell Endocrinol 58:161

Zhang X, Kidder GM, Zhang C, Khamsi F, Armstrong DT 1994 Expression of plasminogen activator genes and enzymatic activities in rat preimplantation embryos. J Reprod Fert 101:235

Zolti M, Meirom R, Shemesh M, Wollach D, Mashiach S, Shore L 1990 Granulosa cells as source and target organ for tumor necrosis factor-alpha. FEBS Letters 261:253

XI. BIBLIOGRAPHY

Julang Li

Education and Degrees Conferred:

Diploma: (Veterinary Medicine)	Foshan Veterinary College, China (1978-1981)
M.Sc. (Physiology):	Changchun Veterinary College, China (1985-1987) Supervisors: Professors Y. Li and Y. Zhang
Ph.D. (Physiology):	University of Ottawa, Canada (1993-1997) Supervisor: Dr. B.K. Tsang

Awards and Scholarships:

Ontario Graduate Scholarship	1995 - 1997
Genesis Research Foundation Studentship	1994 - 1996
Excellence Scholarship, University of Ottawa	1994 - 1997
Admission Scholarship, University of Ottawa	1993 - 1994
Foreign Student Tuition Fee Waiver University of Ottawa	1993 - 1995
Second Prize Award in Science Research Guangdong Province, China	1993
Third Prize of Science and Technology People Liberation Army, China	1988
Graduate Student Award of Excellence Changchun Veterinary College, China	1987
Undergraduate Student Award of Excellence Foshan Veterinary College, China	1981

Publications

I. Refereed Papers (published)

1. Ming Li, **Julang Li**, Liliana Attisano, Jeffrey Wrana, Benjamin K. Tsang (1997) MADR2 expression is up-regulated by TGFβ in hen granulosa cells during ovarian follicular development. Endocrinology 138:3659-3665

2. **Julang Li**, Ming Li, Benjamin K. Tsang (1997) Cytosolic phospholipase A_2 in hen granulosa cells is regulated by transforming growth factor (TGF) alpha and TGF beta during follicular development. Biology of Reproduction 57:929-935

3. **Julang Li**, France Croze, William Yan, Robert Hache, Benjamin K. Tsang (1997) Up-regulation of urokinase plasminogen activator mRNA and protein in hen granulosa cells by TGFα during follicular development. Biology of Reproduction 56:1317-1322

4. **Julang Li**, Daniel L. Simmons, Benjamin K. Tsang (1996) Regulation of hen granulosa cell prostaglandin production by transforming growth factors during follicular development: involvement of cyclooxygenase II. Endocrinology 137: 2522-2529

5. **Julang Li**, Benjamin K. Tsang (1995) Prostaglandins mediate the stimulation of DNA synthesis by transforming growth factor α in hen granulosa cells during follicular development. Biology of Reproduction 52: 1050-1058

6. **Julang Li**, Ming Li, Martine Lafrance, Benjamin K. Tsang (1994) Avian granulosa prostaglandin secretion is regulated by transforming growth factor α and ß and does not control plasminogen activator activity during follicular development. Biology of Reproduction 51: 787-794

7. Jianzhen Huang, **Julang Li** (1993) Effects of stimulating nucleus ambiguous on the ultrastructure changes of insulin cell. Bulletin of Changchun Veterinary College 13(1):40-42.

8. Yeqao Chen, Manxa Huang, **Julang Li** (1991) Interaction of newcastle disease and cryptosporidium infection in chicken. Journal of Foshan Veterinary College (7):5-9.

9. Manxa Huang, **Julang Li**, Yeqao Chen (1990) Histological changes in immune organs of 6chicken with cryptosporidiosis. Journal of Foshan Veterinary College (6): 6-10.

10. **Julang Li**, Yeqao Chen, Manxa Huang (1990) Cryptosporazoon effecting the cellular immunity in chicken. Journal of Foshan Veterinary College (6):11-13.

11. **Julang Li**, Yongtien Li, Yushen Zhang (1988) Nucleus Ambiguous regulating cellular immunity in rabbit. Bulletin of Changchun Veterinary College 8(3):228-233.

12. Yushen Zhang, Changning Liu, **Julang Li**. (1985) Influence of electric stimulus of sometesthesia area of rabbit cerebral cortex on discharge of hyperalgesia neuron in periqueductal grey matter. Bulletin of Changchun Veterinary College 5(2):106-110.

II. Invited Reviews

1. **Julang Li**, Ming Li, Martine Lafrance, Daniel L. Simmons, Benjamin K. Tsang. (1997) Role and regulation of prostaglandin synthesis in the mitogenic response of ovarian granulosa cells to transforming growth factor alpha. In "Eicosanoids and Other Bioactive Lipids in Cancer, Inflammation and Radiation Injury (ed. K.V. Honn, S. Nigam, R. Jones, L.J. Marnette and P.Y.K. Wong) Plenum Publishing Corporation, N.Y. pp509-514

2. Eli G.Karakji, **Julang Li**, Benjamin K. Tsang. (1997) Molecular mechanism of ovulation. *In Vitro* Fertilization and Assisted Reproduction. (Ed. Victor Gomel and Peter C.K.Leung) Mondazzi Editore S.p.A., Bologna, Italy pp241-248.

III. Presentations at Scientific Meetings

1. **Julang Li,** Ming Li, Lilliana Attisano, Jeffrey L. Wrana, and Benjamin K. Tsang. MADR2 expression in avian granulosa cells is up-regulated by TGFβ during ovarian follicular development. 30th Annual Meeting of the Society for the Study of Reproduction, August2-5 1997, Portland, Oregon, USA.

2. Jong-Min Kim, **Julang Li**, Peter Liston, Ming Li, Alexander E. Mackenzie and Robert G. Korneluk, Benjamin K. Tsang. Expression of apoptosis inhibitory proteins (IAPs) in rat granulosa cells during follicular development and atresia. 30th Annual Meeting of the Society for the Study of Reproduction, August2-5 1997, Portland, Oregon, USA.

3. Eli G.Karakji, **Julang Li**, Benjamin K. Tsang. Molecular mechanism of ovulation. 10th World Congress of *In Vitro* Fertilization and Assisted Reproduction. May 24-28 1997, Vancouver, Canada (Invited symposium paper)

4. Benjamin Tsang, Jong-Min Kim, **Julang Li**, Qiangn Feng, Ming Li, David. L Boone, Peter Liston, Alexander E. Mackenzie ane Robert G. Korneluk. Reciprocal Expression of *'Cell Death'* and *'Cell Survival'* Genes in the Induction of Ovarian

Apoptosis: A Pivotal of Gonadotropin. International Meeting on Gastroenterology, Endocrinology and Reproduction, December, 1996, University of Hong Kong, Hong Kong. (Invited symposium paper)

5. Benjamin Tsang, Jong-Min Kim, **Julang Li**, Qiang Feng, Ming Li, David. L Boone, Peter Liston, Alexander E. Mackenzie and Rober G. Korneluk. Regulation of Ovarian Follicular Development: Basic Concepts and Clinical Implications. 42nd Annual Meeting of the Canadian Fertility and Andrology Society, November,1996, Lake Louise, Alberta. (Invited symposium paper)

6. **Julang Li**, Benjamin K. Tsang. Cytosolic phospholipase A_2 in hen granulosa cells is up-regulated by transforming growth factor (TGF) alpha and suppressed by TGF beta during follicular development. 29th Annual Meeting of the Society for the Study of Reproduction, July 1996, London, Ontario, Canada.

7. **Julang Li**, Ming Li, Benjamin K. Tsang. Cytosolic phospholipase A_2 in hen granulosa cells is up-regulated by transforming growth factor (TGF) alpha and suppressed by TGF beta during follicular development. 15th Annual Ottawa Reproductive Biology Workshop, June, 1996, Ottawa, Ontario, Canada.

8. **Julang Li,** Daniel L. Simmons, Benjamin K. Tsang. Regulation of prostaglandin production by transforming growth factors in granulosa cell mitogenic response: role of cyclooxygenase II. 4th International Conference on Eicosanoids & other Bioactive Lipids in Cancer, Inflammation & Radiation Injury, October, 1995, Hong Kong.

9. **Julang Li,** Daniel L. Simmons, Benjamin K. Tsang. Cyclooxygenase II in hen granulosa cells is regulated by transforming growth factors during follicular development. 14th Annual Ottawa Reproductive Biology Workshop, June, 1995, Ottawa, Ontario, Canada.

10. **Julang Li,** Daniel L. Simmons, Benjamin K. Tsang. Cyclooxygenase II in hen granulosa cells is regulated by transforming growth factors during follicular development. 41st Annusl Meeting of the Canadian Fertility and Andrology Society, September, 1995, Montebello, Quebec.

11. **Julang Li** , Benjamin K. Tsang. The mitogenic effect of TGFα is mediated by prostaglandins in hen granulosa cells during ovarian follicular development. Xth Ovarian Workshop, July 1994, Ann Arbor, Michigan, U.S.A.

12. **Julang Li** , Benjamin K. Tsang. Prostaglandins are mediators of TGFα-induced avian granulosa cell DNA synthesis. XIIIth Annual Ottawa Reproductive Biology Workshop, June, 1994, Ottawa, Canada.

13. Martine Lafrance, Ming Li, **Julang Li**., Benjamin K. Tsang. Regulatory effects of transforming growth factor alpha and beta-1 on prostaglandin secretion by avian granulosa cells during follicular development. 26th Annual Meeting of the Society for the Study of Reproduction, August 1993, Fort Collins, Colorado, U.S.A.

14. Martine Lafrance, Ming Li, **Julang Li**, Benjamin K. Tsang. Regulatory effects of transforming growth factor alpha and beta-1 on prostaglandin secretion by avian granulosa cells during follicular development. XIIth Annual Ottawa Reproductive Biology Workshop, June 1993, Ottawa, Canada.