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REVIEW

Recent Discoveries in Physiology of Insulin-like Growth Factor-1 and Its Interaction with Gonadotropins in Folliculogenesis

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Ovarian follicular development is under the influence of gonadotrophins. The manner by which gonadotrophins achieve their role in cell replication and hormone production is through other specialized molecules. Insulin-like growth factor-1 (IGF-1) is considered to be one of the most important of these molecules. IGF-1 is present in relatively large amounts in the ovary and the IGF-1 gene is expressed in the graafian follicle. IGF-1 binding proteins modulate the action of IGF-1 and are influenced by gonadotrophins. Null mutations of genes encoding IGF-1 and type-1 IGF receptor have demonstrated the obligatory role of IGF-1 in folliculogenesis. It was proposed that IGF-1 may be an *obligatory* mediator of gonadotrophin-induced folliculogenesis and that the interaction is *synergistic*.

From this point, one group embarked on a series of physiological studies to further elucidate the interaction of these molecules. We discovered that, according to circumstances, follicle stimulating hormone (FSH) may act with IGF-1 *synergistically* (as was postulated before), *additively, independently,* or even *antagonistically*. We discovered that granulosa cells must first be exposed to FSH and then they respond well to IGF-1. All previous studies of granulosa cells were done on mural granulosa cells. We subsequently showed that cumulus type of granulosa cells (adjacent to the oocyte) behave differently in their responses to gonadotrophins and IGF-1.

Key Words: Insulin-like growth factor-1; follicle-stimulating hormone; luteinizing hormone; granulosa cells; cumulus cells; DNA synthesis.

Introduction

Insulin-like Growth Factor-1

Folliculogenesis is the process by which the somatic cells surrounding dormant oocytes, collectively constituting the pool of primordial follicles present in the ovary at birth, begin to grow and differentiate, ultimately forming the mature follicles capable of ovulation. The physiologic role of follicle-stimulating hormone (FSH) in both the regulation of follicle growth and the differentiation of granulosa cells in vivo is well established. The mechanism of stimulation of differentiated functions by FSH, such as induction of steroidogenic enzymes and luteinizing hormone (LH) receptors, is shown to be related to specific binding of these hormones to granulosa cell membrane receptors causing an increase in intracellular levels of cyclic adenosine monophosphate (cAMP) mediating the cellular responses to the hormone *(1,2)*. However, the mechanism by which FSH stimulates follicle growth is not well understood, and attempts to demonstrate direct mitogenic effects of FSH on isolated granulosa cells have been unrewarding. Not withstanding, several growth factors such as insulin-like growth factor-1 (IGF-1) have been shown to have a marked effect on granulosa cell proliferation in vitro *(3,4)*.

In a discussion of granulosa cells, one must be aware that the ovarian follicle contains different types of granulosa cells at the antral stage. There is regional differentiation with respect to morphology, steroidogenic enzyme activity, and gonadotropin receptor populations, from the peripheral (mural) granulosa cells to those in closest proximity to the oocyte (cumulus cells [CCs] and corona radiata cells) *(5)*.

The molecular biology and mechanism of growth promotion of IGF-1 have been studied extensively in other well-characterized cell systems, such as the Balb/C 3T3 cell line. In stimulating mitosis, IGF characteristically acts in concert with other growth factors, notably basic fibroblast growth factor, platelet-derived growth factor, and epidermal growth factor (EGF), each acting at a different point in the cell cycle *(6)*. Cooperation among these growth factors has been reported in stimulating porcine granulosa cell proliferation in culture *(7)*.

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IGF-1 binds to various binding proteins, which are thought to antagonize the action of IGF-1. In turn, the proteases, which inactivate the binding proteins, result in IGF-1 being free and biologically active *(8)*.

IGF-1 Gene Expression and Its Presence in Graafian Follicle

Of all adult organs tested in the rat (the model most frequently used), the liver and uterus are the most active but the ovary ranks third in IGF-1 presence and its gene expression *(9)*. This may indicate that the locally produced, rather than the circulatory, IGF-1 is responsible for IGF-1 action in the ovary. It has been established through cellular localization studies that the granulosa cells are the sole somatic ovarian cells concerned with IGF-1 gene expression *(10–12)* and translation *(13)*. FSH has been reported to increase IGF-1 gene expression and production in granulosa cells of several species *(14)*. Therefore, this has raised the possibility that the follicle-stimulating action of FSH is mediated via IGF-1, which will be acting in a paracrine or autocrine manner. IGF-1 is present in porcine follicular fluid *(15)* and is secreted by porcine granulosa cells when exposed to gonadotropins *(16)*. Prepubertal gilts treated with equine chorionic gonadotropin (eCG) demonstrated an increase in follicular but not the plasma IGF-1 level *(8)*. These results indicate that the increase in follicular IGF-1 is owing to local IGF-1 production rather than passive entrance into the follicle from general circulation *(17)*.

Role of IGF-1 in Ovarian Steroidogenesis

It has been shown that the androgen biosynthesis of thecal interstitial cells (TICs) is not influenced by IGF-1 *(18, 19)*. LH has a marked stimulatory effect on TIC androgen production. IGF-1 in combination with LH causes a further three- to fivefold stimulation of androgen production beyond that of LH alone. Hence, IGF-1 causes a synergistic augmentation of androgen production *(20–22)*. IGF-1 has been shown to further the response of TICs to LH by increasing the number of LH receptors and by augmenting the intracellular response to the LH stimulus *(23–24)*. IGF-1 enhances lipoprotein metabolism, which results in a rise in the intracellular pool of cholesterol. In turn, this is available for enhanced steroidogenesis *(22–24)*. Concentration of steroidogenic enzymes present in mitochondria and microsomes are increased in response to IGF-1 *(19,25–27)*.

IGF-1-Binding Proteins

The rat ovary has been demonstrated to be a highly compartmentalized (hormonally dependent) site of expression of IGF-binding proteins (IGFBPs) 2–6 but not IGFBP 1 *(28)*. IGFBP 4 and IGFBP 5 were shown to be selective for granulosa cells *(28–30)*. On the other hand, IGFBPs 2, 3, and 6 are exclusively shown in TICs *(31–34)*. The presence of IGFBPs 4 and 5 was noted to be poorly expressed in healthy antral follicles, whereas they were well expressed in atretic follicles *(28–30)*. Atretic follicles as well show low levels of IGF-1 gene expression *(10)*. In cultured rat granulosa cells, exogenously added IGFBPs 1–5 attenuated the FSH-supported steroidogenesis *(35–37)*. In immature rats primed with pregnant mare's serum gonadotropin, intrabursal administration of IGFBP 3 resulted in attenuation of human chorionic gonadotropin (hCG)-triggered ovulation *(38)*. The elaboration of IGFBP 5 was noted to be inhibited by FSH in cultured rat granulosa cells. It has been shown that the bioactivity of IGFBP 4 is determined by a specific metalloprotease that is expressed by granulosa cells. This protease cleaves the 24-kDa protein into smaller fragments that do not bind IGF-1 with high affinity *(39)*. Mature IGFBP 4 blocks FSH-dependent granulosa cell differentiation *(40)*. Therefore, control of IGFBP 4 protease expression may have a significant role in follicular apoptosis and atresia. High concentrations of FSH were demonstrated to induce IGFBP 4 protease activity. This may be an indirect manifestation that FSH influences IGF-1 availability *(40–42)*. Figure 1 depicts the interaction of these factors schematically. It has been demonstrated that physiologic concentrations of IGF-1 stimulate the secretion of IGFBPs 2 and 4 in TIC culture environments *(43)*.

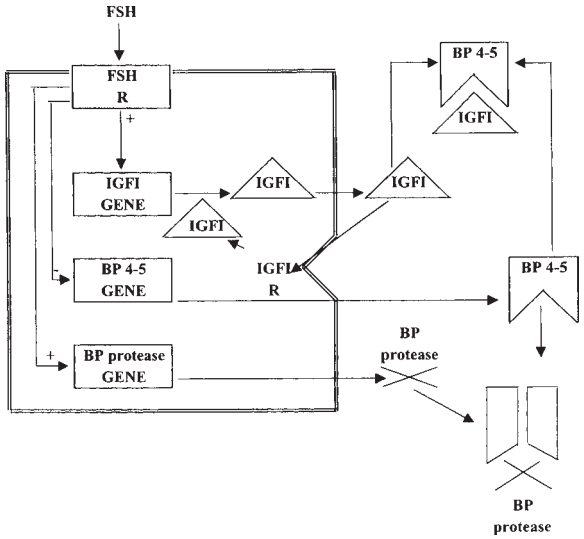

Fig. 1. Interaction of FSH, FSH receptor, IGF-1 gene, IGF-1 receptor, IGF-1, IGFBPs 4 and 5 genes, IGFBPs 4 and 5, BP protease gene, and BP protease. The inside (in the double line) and outside of the cell are shown.

Preparation of Isolated Perfused Rabbit Ovary

Ovulation failed to occur in both control and experimental ovaries that were perfused with IGF-1 in varying concentrations in the absence of gonadotropins. Exposure to IGF-1 stimulated the secretion rate of angiotensin II–like immunoreactivity (Ang II-IR) in perfused rabbit ovaries in a dose-dependent manner. The percentage increase in follicle diameter in ovaries perfused with IGF-1 for 12 h was significantly correlated with the secretion rate of Ang II-IR. Exposure to IGFBP 3 inhibited hCG-induced ovulation.

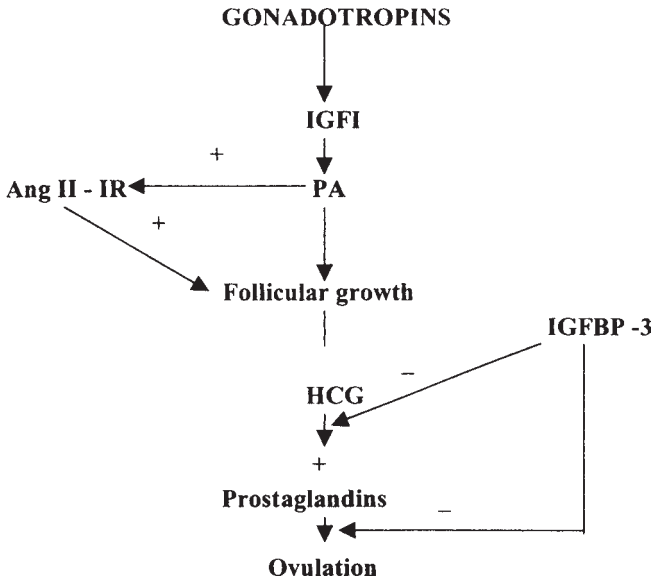

Fig. 2. Deductions from isolated perfused rabbit ovary model.

Intrafollicular plasminogen activator activity significantly increased within 4 h after exposure to 100 µg/mL of IGF-1. In vivo exposure to streptokinase, an exogenous plasminogen activator, stimulated both follicular growth and intrafollicular Ang II-IR content. IGF-1 enhanced both ovarian Ang II production and follicular development by stimulating intrafollicular plasminogen activator activity *(44)*. The deduction from this experiment is shown schematically in Fig. 2.

Null Mutations of Genes Encoding IGF-1 and Type 1 IGF Receptor

Newborn mice homozygous for a targeted disruption of IGF-1 gene exhibited growth deficiency. While a percentage of dwarf mice die shortly after birth, others survive and reach adulthood. The female mutants fail to ovulate even after administration of gonadotropins, which is apparently the primary cause of their infertility. These mice possess an infantile uterus that exhibits a dramatic hypoplasia. Although some IGF-1 null mutants exhibited mating behavior, they were infertile owing to lack of ovulation. IGF-1 was found not to be obligatory in follicular development up to the formation of antral follicles. After a surge of exogenously administered gonadotropins, follicular responses culminating in rupture of the follicle did not occur *(45)*. By contrast, null mutants for IGF-1 gene receptor invariably die at birth of respiratory failure and exhibit more severe growth deficiency.

Effect of IGF-1 on Oocyte Maturation, Fertilization, and Early Embryonic Development

The cleavage rate of oocyte maturation was markedly stimulated by the addition of IGF-1, to the oocyte maturation medium. In addition, embryonic development was improved by IGF-1. These experiments, which were conducted on the porcine model, indicated a beneficial effect of IGF-1 on in vitro oocyte maturation and preimplantation embryonic development *(46)*. The deduction from this study is shown schematically in Fig. 3.

A Comparative Study of Mural and Cumulus Granulosa Cells

Recent findings illustrate that there are two distinct types of granulosa cells: CCs, which surround the oocyte, and mural granulosa cells (MGCs), which are found around the antrum of the follicle. This is a specialization of the cells, probably related to the interaction of CCs with the oocyte, whereas the MGCs are more distant from the oocyte and more involved with the formation of corpus luteum. The CC provides nutrition and other materials for the oocyte through direct connecting channels *(47)*. Recently, it has been shown that the oocyte influences its surrounding CCs *(48)*. Therefore, a unit is formed consisting of the oocyte and its surrounding cells, which is termed the oocyte cumulus complex (OCC). The specialized type of granulosa cells surrounding the oocyte (CCs) are approx 1000 (10^3) in number. Therefore, an OCC consists of one oocyte and approx 1000 CCs. It should be noted that the majority of studies to date have utilized MGCs. In addition, granulosa cells were from immature rats treated with estrogen. This represents a rather unphysiologic setup, if the interest is in maturing follicles involved in ovulation (see review in ref. *49*).

To address these issues, research was designed to evaluate the interaction of gonadotropins, estradiol, and IGF-1 on proliferation, differentiation, and progesterone production of bovine OCCs and MGCs *(50)*. Ovaries obtained from cows at the time of slaughter were held at 25–30ºC in phosphate-buffered saline while transported to the laboratory. Within a 2-h period, follicles of 1–5 mm in diameter were aspirated with syringes containing culture medium. OCCs and MGCs were identified under a microscope and were separately cultured in serum-free medium. CCs and MGCs are easily distinguished since the CCs have a distinct shiny appearance related to their mucification. The majority of CCs were seen attached to the oocyte. Ten to twenty OCCs consisting of approx 2.5×10^5 CCs or 2.5×10^5 MGCs per well were cultured with varying concentrations of recombinant human IGF-1, EGF, purified porcine FSH, purified porcine LH, or estradiol-17β, or with a specified combination of these. After 18 h of culture, ^{3}H-thymidine was added, and cultures were continued for 6–8 h to permit labeling of DNA as an index of cell proliferation, based on the rate of DNA synthesis. Cell differentiation was assessed by evaluating the expansion of CCs and steroidogenic activity of both OCCs and MGCs.

The results were significantly different when comparing OCCs and MGCs. There were small (2.5-fold) but significant increases noted in CC replication in response to both FSH and LH. FSH but not LH also stimulated MGC

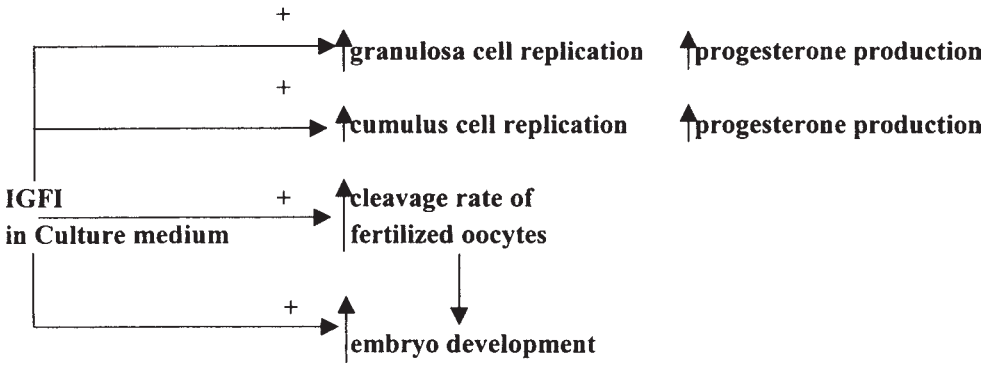

Fig. 3. Effect of IGF-1 on oocyte maturation, fertilization, and early embryonic development in porcine model *(46)*.

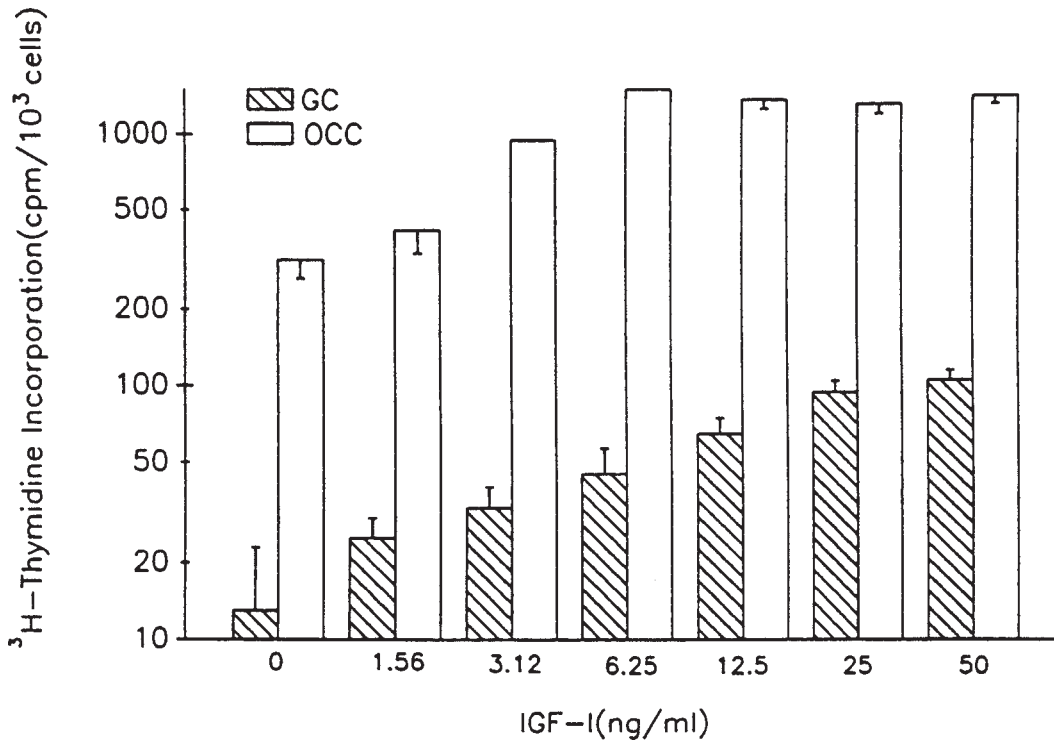

Fig. 4. Effects of increasing concentrations of IGF-1 on ^{3}H-thymidine incorporation in CC complexes and granulosa cells from bovine small follicles during 24-h culture in vitro. Each OCC consists of an oocyte surrounded by approx 10^3 CCs. Comparisons were made as events per each 1000 cells. Each follicle yielded one OCC. Cells from different follicles were pooled. Cumulus type of granulosa cells were in a compact form around the oocyte, whereas mural granulosa cells (GC) were in small clumps. [Reprinted with permission from the Society for the Study of Reproduction (*Biol. Reprod.* 1996; **54,** 331–338).]

replication from small antral follicles. IGF-1 caused a higher cell replication both for OCCs (18-fold) and MGCs (6-fold). It was noted that cell replication was higher in OCCs vs MGCs, both with and without FSH. Calculations were done per each CC vs MGC. Each OCC was calculated to contain approx 1000 cells. Figure 4 demonstrates the relationship between DNA synthesis and dose of IGF-1 in the culture medium. It can be seen that the dose-response curves for OCC were very different from those of MGCs. CCs responded maximally at 6–12 ng/mL of IGF-1, whereas the response of MGCs continued to increase up to the maximum concentration used (50 ng/mL). Various combinations of FSH and IGF-1 were used in a subsequent study, and the results showed a startling difference between CCs and MGCs. MGCs showed an additive effect of FSH and IGF-1, whereas FSH was highly inhibitory of the proliferative response of CCs to IGF-1 (Table 1). Further research depicts the same findings (Figs. 5 and 6). Figure 5 shows the results of interaction of FSH and IGF-1 with respect to the CCs. Note that while IGF-1 causes increased replication, the addition of FSH dramatically reduces cell replication. Figure 6 shows the same setting for MGCs that do not manifest this antagonistic effect of FSH on IGF-1-induced cell replication.

Two possible mechanisms for the aforementioned observations were investigated: (1) whether the previously demonstrated inhibitory effect of FSH on DNA synthesis could

Table 1
Effects of Gonadotropins and Growth Factors on ^{3}H-Thymidine Incorporation in Bovine CCs and Granulosa Cells In Vitro *(50)*[a]

	^{3}H-Thymidine incorporation	
In vitro treatment	CCs (cpm/OCC)	Granulosa cells (cpm/10^3 cells)
Control	890 ± 115*	8.2 ± 2.4*
IGF-1 (50 ng/mL)	10,543 ± 1736†	35.7 ± 0.7†
FSH-V001 (100 ng/mL)	1233 ± 136‡	15.2 ± 4.7*
IGF-1 + FSH	1725 ± 109‡	43.6 ± 3.3†

[a] Values are the mean ± SEM from three separate experiments; $n = 3$ in each experiment. Different superscripts in the same column indicate statistical difference at $p < 0.05$. [Reprinted with permission from the Society for the Study of Reproduction (*Biol. Reprod.* 1996; **54,** 331–338).]

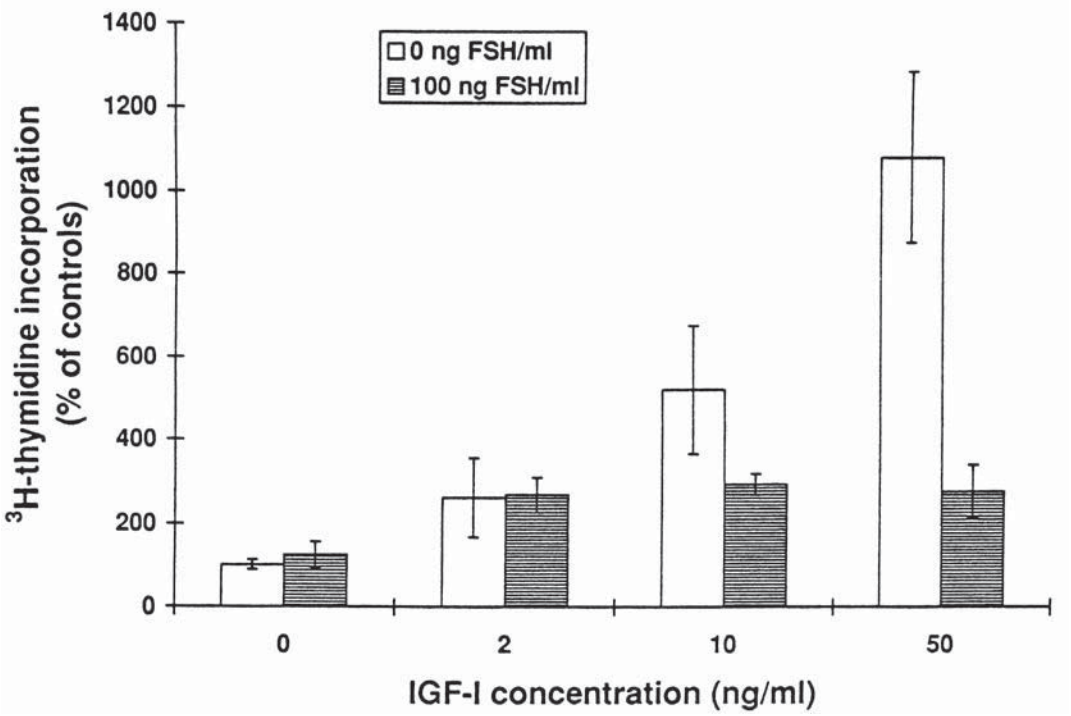

Fig. 5. Effect of IGF-1 and FSH on DNA synthesis in bovine OCCs. Plotted values are mean ± SEM for pooled data from three separate experiments, normalized to percentage of controls (without FSH or IGF-1). Mean percentages for separate experiments ($n = 3$) were analyzed by two-way analysis of variance (ANOVA). IGF-1 concentration effects and FSH effects were significant ($p < 0.05$), with significant FSH × IGF-1 interaction ($p < 0.05$). [Reprinted with permission from the Society for the Study of Reproduction (*Biol. Reprod.* 1997; **57,** 684–688).]

be related to its ability to cause CC mucification as a differentiated response, incompatible with continued proliferation; and (2) whether increased IGFBPs that were secreted in response to FSH then competed with IGF receptors, thus inhibiting response to exogenous IGF-1. To determine the effects of cumulus mucification in modulating the mitogenic response to IGF-1, two other agents that induce cumulus mucification by different mechanisms, EGF and dibutyryl cAMP (dbcAMP), were compared with FSH (Tables 2 and 3). To determine the possible role of IGFBP in modulating the mitogenic response to IGF-1, an IGF-1 analog that does not bind to IGFBP, long arg^3-IGF-1 (LR^3-IGF-1), was compared with native IGF-1 for efficacy in stimulating DNA synthesis in the absence and presence of each of the agonists (Fig. 7 and Tables 2 and 3). Both IGF-1 and LR^3-IGF-1 stimulated ^{3}H-thymidine incorporation in CCs to a much greater extent than in MGCs. Incorporation in mural cells was increased by each of FSH, EGF, and dbcAMP acting individually. In most instances, this result was considerably enhanced by the combined action of each of these agents with each of the IGF-1 forms. By contrast, the significantly greater stimulatory effect of both IGF-1 and LR^3-IGF-1 on

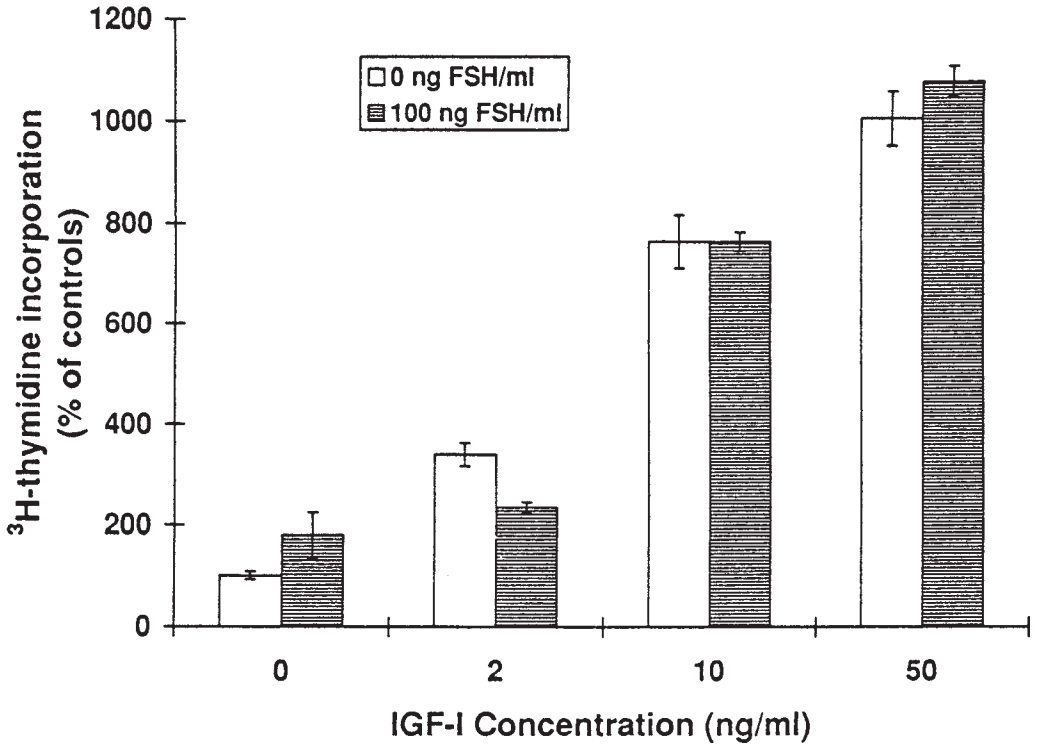

Fig. 6. Effect of IGF-1 and FSH on DNA synthesis in bovine MGCs. Plotted values are mean ± SEM for pooled data from three separate experiments, normalized to percentage of controls (without FSH or IGF-1). Mean percentages for separate experiments ($n = 3$) were analyzed by two-way ANOVA. IGF-1 concentration effects were significant ($p < 0.05$); FSH effects and FSH × IGF-1 interaction were not significant ($p > 0.05$). [Reprinted with permission from the Society for the Study of Reproduction (*Biol. Reprod.* 1997; **57,** 684–688).]

Table 2
Effects of FSH, EGF, and dbcAMP Without and With IGF-1 on ^{3}H-Thymidine Incorporation in Bovine CCs (means ± SEM)[a]

	dpm per OCC[b]		
Modulating agent	Control	IGF-1 (10 ng/mL)	LR^3-IGF-1 (10 ng/mL)
None	1475 ± 53*	12,240 ± 3586*	19,360 ± 1960*
FSH (100 ng/mL)	2782 ± 292†	4599 ± 391†	5265 ± 492†
EGF (5 ng/mL)	2750 ± 292†	3696 ± 451†	5301 ± 954*
dbcAMP (2.5 μ*M*)	3044 ± 329†	4462 ± 147†	3406 ± 431†

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[b]Two-way ANOVA indicated highly significant between-row and between-column effects ($p < 0.001$), and row × column interactions ($p < 0.001$); values within the same column with different superscript symbols are significantly different ($p < 0.05$). OCC represents one oocyte plus approximately 10^3 CCs.

Table 3
Effects of FSH, EGF, and dbcAMP Without and With IGF-1 on ^{3}H-Thymidine Incorporation in Bovine MGCs (means ± SEM)[a]

	dpm per 10^3 granulosa cells[b]		
Modulating agent	Control	IGF-1 (10 ng/mL)	LR^3-IGF-1 (10 ng/mL)
None	19 ± 5*	21 ± 2*	18 ± 3*
FSH (100 ng/mL)	26 ± 6†	49 ± 10†	40 ± 13†
EGF (5 ng/mL)	105 ± 8†	112 ± 16 †	321 ± 130*
dbcAMP (2.5 μ*M*)	227 ± 3†	683 ± 146†	332 ± 20†

[a]Reprinted with permission from the Society for the Study of Reproduction (*Biol. Reprod.* 1997; **57,** 684–688).

[b]Two-way ANOVA indicated highly significant between-row ($p < 0.001$) and between-column ($p < 0.02$) effects, and row × column interactions ($p < 0.001$); values within the same column with different superscript symbols are significantly different ($p < 0.05$).

CCs was markedly decreased by each of FSH, EGF, and dbcAMP. These findings suggest that the inhibition of IGF-1-stimulated DNA synthesis in CCs is a consequence of induction of CC differentiation (mucification) by FSH and EGF rather than through competition between IGF-1 receptor and IGFBP secretion induced by these agents (Fig. 8).

Studies in the same bovine model *(50)* revealed that a concentration of 25 ng/mL of IGF-1 caused a fivefold increase in cell replication in MGCs when a dose-response curve experiment was carried out (IGF concentration of 0–50 mg/L). An experiment was subsequently conducted in order to assess cell replication vs the varying dose of FSH

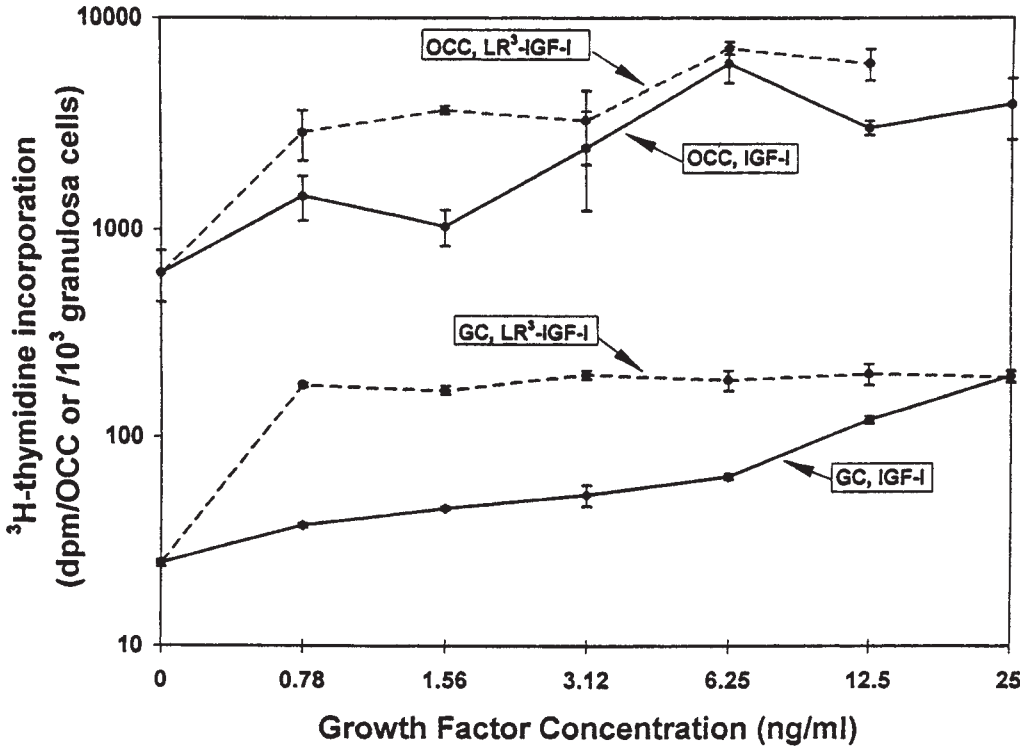

Fig. 7. Effect of increasing concentrations of IGF-1 and LR^3-IGF-1 on DNA synthesis in bovine OCCs and mural granulosa cells (GC). Plotted values are mean ± SEM dpm incorporation of triplicate determinations at each growth factor concentration. Two-way ANOVA indicated that the effects of IGF-1 and LR^3-IGF-1 differed significantly for both cell types ($p < 0.001$), with significant growth factor × concentration interaction ($p < 0.001$) only for mural granulosa cells. [Reprinted with permission from the Society for the Study of Reproduction (*Biol. Reprod.* 1997; **57,** 684–688).]

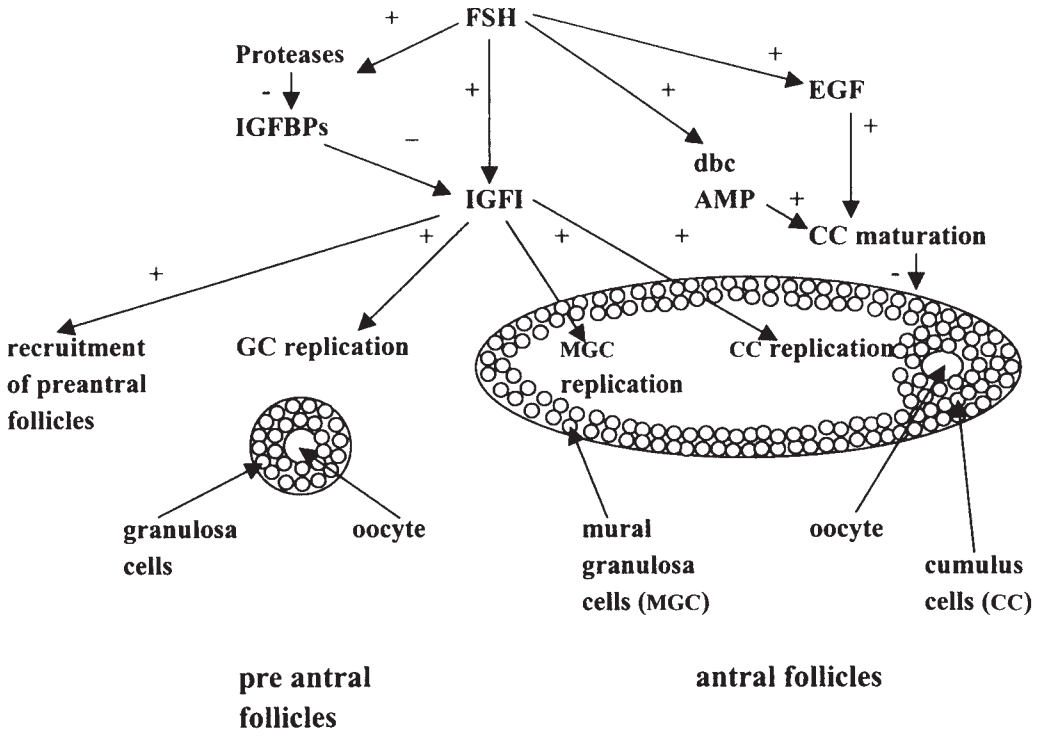

Fig. 8. Proposed model for interaction of FSH and IGF-1 in folliculogenesis *(51)*.

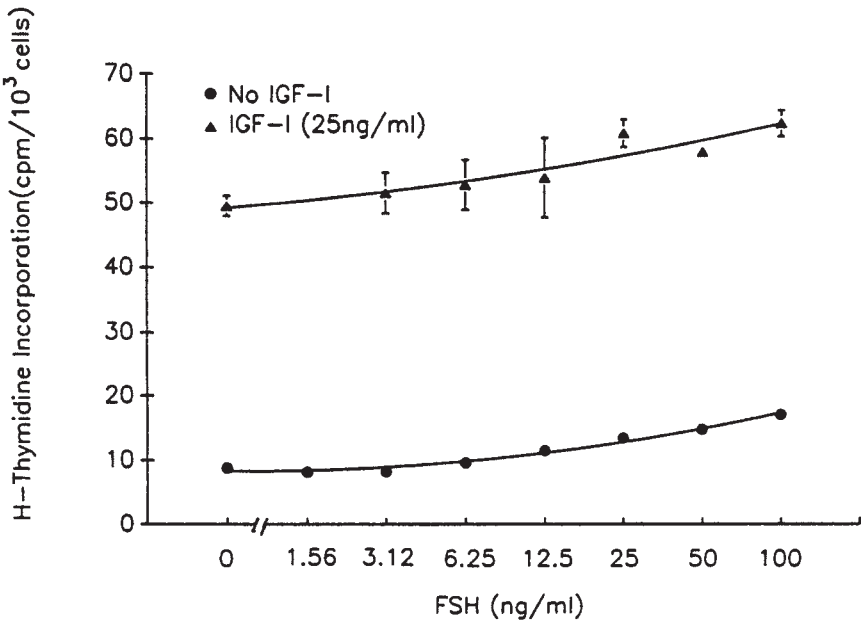

Fig. 9. Effects of increasing concentrations of FSH in absence and presence of 25 ng/mL of IGF-1 on ^{3}H-thymidine incorporation in bovine granulosa cells during 24-h culture in vitro. [Reprinted with permission from the Society for the Study of Reproduction (*Biol. Reprod.* 1996; **54,** 331–338).]

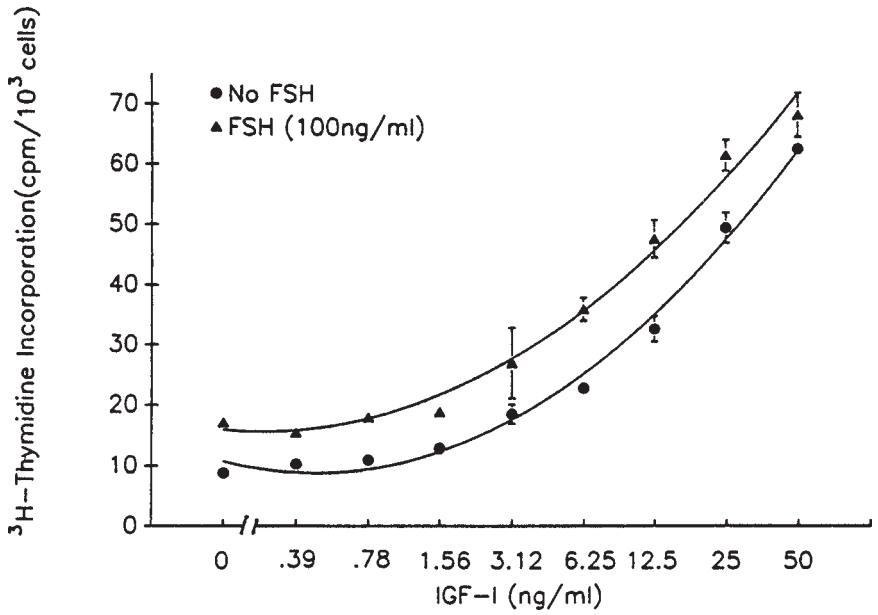

Fig. 10. Effects of increasing concentrations of IGF-1 in absence and presence of 100 ng/mL of FSH on ^{3}H-thymidine incorporation in bovine granulosa cells during 24-h culture in vitro. [Reprinted with permission from the Society for the Study of Reproduction (*Biol. Reprod.* 1996; **54,** 331–338).]

from 0 to 100 ng/mL (Fig. 9). The cell replication almost doubled in this range of treatment for MGCs. A similar dose-response curve was obtained with the addition of a fixed amount of IGF-1 (25 ng/mL) to each dose of FSH. Additions of IGF-1 caused a fivefold increase beyond the effect of FSH at each dose level of FSH. The two dose-response curves of FSH vs cell replication and FSH plus fixed dose of IGF-1 vs cell replication were parallel. This indicated that the effect of IGF was additive and not synergistic in this situation.

Similarly an experiment was conducted *(50)* to obtain a dose-response curve of IGF-1 dose (0–50 ng/mL) vs cell replication for MGCs (Fig. 10). A second dose-response curve was obtained by the addition of a fixed dose of FSH (100 ng/mL) to each dose of IGF-1. IGF-1 was noted to have a dramatic effect on MGC replication, rising sixfold from a dose of 0 to 50 ng/mL. FSH demonstrated a constant positive effect. The cell replication at each dose level was doubled. It can be concluded that FSH had an additive effect on IGF-1-induced cell replication since the two curves were

Table 4
Effect of Pre- and Cotreatment with IGF-1 or EGF on Progesterone Secretion by CCs and Granulosa Cells in Response to FSH[a]

In vitro treatment		Progesterone secretion[b]	
0–8 h	8–28 h	CCs (pg/OCC)	Granulosa cells (pg/10^3 cells)
Control	Control	169 ± 37*	15.9 ± 0.5*
IGF-1 (50 ng/mL)	IGF-1	200 ± 12*	37.8 ± 2.7†
EGF (5 ng/mL)	EGF	154 ± 8*	16.2 ± 1.1*
FSH (100 ng/mL)	FSH	189 ± 45†	19.6 ± 1.1*
Control	FSH	126 ± 26†	13.8 ± 0.2*
IGF-1	IGF-1 + FSH	379 ± 74†	41.8 ± 5.6†
EGF	EGF + FSH	201 ± 11†	15.6 ± 0.9*
IGF-1 + FSH	IGF-1 + FSH	460 ± 80†	46.2 ± 1.4†

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[b]Values represent arithmetic means ± SEM of untransformed data from three replicates per treatment. Values with different superscript symbols, within columns, are significantly different ($p < 0.05$) on the basis of ANOVA of logarithmically transformed data.

Table 5
Effects of FSH and IGF-1 Alone and in Combination on Progesterone Secretion by Bovine CCs and Granulosa Cells In Vitro[a]

	Progesterone secretion[b]	
Treatment	CCs (pg/OCC)	Granulosa cells (pg/10^3 cells)
Control	73 ± 11*	27 ± 4*
IGF-1 (50 ng/mL)	210 ± 21†	48 ± 2†
FSH-V001 (200 ng/mL)	196 ± 27†	36 ± 1†
IGF-1 + FSH	297 ± 23‡	89 ± 4‡

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[b]Values represent arithmetic means ± SEM of untransformed data from three replicates per treatment. Values with different superscript symbols, within columns, are significantly different ($p < 0.05$) on the basis of ANOVA of logarithmically transformed data. OCC represents one oocyte and approx 1000 surrounding CCs.

parallel. In this situation, they did not act synergistically, which would have necessitated the lines diverging. This was an important observation because it was believed that IGF-1 and FSH always act synergistically.

Differentiation response of granulosa cells may be assessed by CC expansion or progesterone secretion. In experiments using a bovine model *(50)*, it was found that FSH was highly effective in inducing CC expansion but IGF-1 did not cause such maturation. Progesterone secretion appeared to respond quite sluggishly to FSH in both OCCs and MGCs. IGF-1 alone caused a significant (approximately twofold) increase in progesterone secretion by MGCs but not OCCs. A synergistic effect (at lower dose of FSH) and an additive effect (at higher dose of FSH) were noted when FSH and IGF-1 were used together to study progesterone production (Tables 4 and 5).

Table 6
Effect of In Vivo Estrogen Therapy on ^{3}H-Thymidine Incorporation of MGC[a]

	^{3}H-Thymidine incorporation of MGCs[b]	
	Control in vivo	Estrogen in vivo
Control in vitro	26 ± 0.7*	15 ± 0.6*
IGF-1 in vitro	81 ± 4†	18 ± 2*

[a]Reprinted by permission from the American Society for Reproductive Medicine (*Fert. Ster.* 2001; **75,** 997–1002).

[b]Different superscript symbols signify statistically significant difference ($p < 0.01$), in the same column (between control and IGF-1).

Comprehensive Physiological Studies

An understanding of the interaction of gonadotropins and IGF-1 has been affected by two obstacles. First, most studies have not taken into consideration the difference between OCCs vs MGCs. The cell replication by CCs is many folds higher than by MGCs. The CCs are under the influence of the oocyte. These cells undergo cumulus expansion and remain in proximity of the oocyte after ovulation. Second, in the rat model, immature animals were treated with estrogen, which is shown to cause immature granulosa cell proliferation *(31)*, whereas our physiologic interest is in maturing or mature granulosa cells associated with ovulation or superovulation. Estrogen therapy of immature rats will shut off FSH production by the pituitary gland. Therefore, the granulosa cells of such rats have had very little exposure to FSH. We evaluated the situation in two experiments *(52)*. In one, the effect of in vivo estrogen therapy was investigated vs no estrogen therapy. It was shown that the MGCs from animals with estrogen therapy showed no response to IGF-1 in vitro (Table 6). In another set of experiments, premature rats were infused with FSH containing compounds such as eCG vs saline. In each group, OCCs and MGCs were obtained in vitro and subjected to either saline or varying doses of IGF-1. Each OCC consisted of one oocyte with approx 10^3 CCs. It was shown that MGCs responded more vigorously to IGF-1 (vs animals not exposed to) as judged by ^{3}H-thimidine-incorporated studies (an indicator of cell replication) (results are shown in Fig. 11).

Previously it was recorded that rat granulosa cells did not show increased cell replication when exposed to IGF-1, whereas MGCs from other species showed a vigorous response to IGF-1 *(49,50)*. A possible explanation is that prior exposure to FSH is obligatory for granulosa cells to respond to FSH. Prior exposure to FSH allows follicles to grow at the antral level where rapid replication of granulosa cells

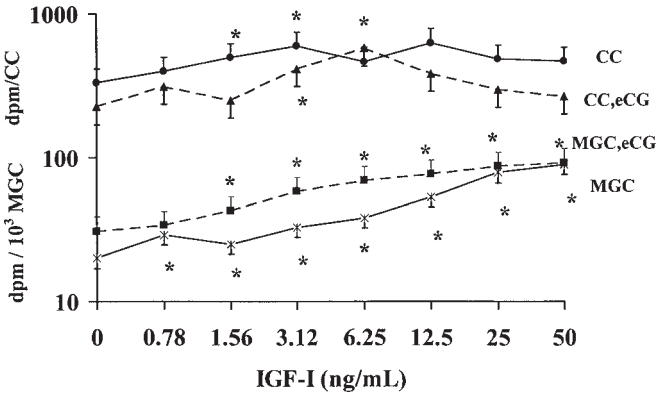

Fig. 11. Effect of IGF-1 in vitro and eCG in vivo on ^{3}H-thymidine incorporation during 28-h culture of MGCs and oocyte-cumulus complexes (CC) of immature rats. Data are mean values ± SEM for pooled data from two experiments, with six in vitro replicates per IGF-1 concentration. (—●—), CCs from saline-treated animals; (—▲—), MGCs from eCG-treated animals; (—X—), MGCs from animals treated with saline; (*), values significantly different from control (no IGF-1), within the same cell type and in vivo treatment ($p < 0.05$). Reprinted with permission from the American Society for Reproductive Medicine (*Fert. Ster.* 2001; **75,** 997–1002).

occur. We may imagine that FSH acts as the key to turn on the switch for the granulosa cell replication, which is then greatly influenced by locally produced IGF-1. FSH has been shown to induce expression of IGF-1 receptor, which is probably the explanation for the latter observations.

Immature rats were treated with either gonadotropin (eCG) or saline. Forty-eight hours later they were sacrificed and the ovaries were removed *(52)*. Surface follicles (approx 30 follicles per rat) on the ovaries were punctured with a hypodermic needle and OCCs and MGCs were removed and cultured. A dose-response curve was obtained by culturing the OCCs and MGCs with varying concentrations of IGF-1 (from 0.78 to 50 mg/mL). ^{3}H-Thymidine incorporation was used as an indicator of DNA synthesis and cell replication; the experimental results are shown in Fig. 11. The two variations of in vivo treatments with or without gonadotropins and in vitro exposures to varying dose of IGF-1 revealed the following results: First, MGCs with no in vivo treatment with gonadotropin showed a gradual increase in cell replication reaching fourfold at the highest level vs the 0 IGF-1 dose level. Second, OCCs with no in vivo gonadotropins demonstrated the basal rate of cell replication of CCs (with no added IGF-1) to be sixfold higher in comparison with MGCs. Analyzed per cell, the response of OCCs to IGF-1 was much less than MGCs, reaching a maximum twofold increase. At the higher dose levels, there was plateauing or even a decline. Third, MGCs, treated in vivo with gonadotropin, showed a gradual and dose-dependent positive response to increasing concentration of IGF-1. This plateaued at 12.5 mg/mL. In vivo (prior) treatment with gonadotropins resulted in a statistically significant increase in cell replication up to an IGF-1 dose of 25 µg/mL. Fourth, OCC treatment in vivo with gonadotropins showed an increase in incorporation up to 6.25 ng/mL of IGF after which it declined. This curve did not show any persistent difference from when no gonadotropins were used in vivo *(52)*.

To understand the physiological or pharmacological role of IGF-1 in reproduction, this compound has to be injected into living animals. However, when IGF-1 is administered, it is bound to several binding proteins that modulate the action of IGF-1. We have had access to an analog of IGF-1 (LR^3-IGF-1) that binds very poorly to the binding proteins. Therefore, we were able to conduct experiments when the effect of IGF-1 could be assessed without its modulation by the binding proteins.

We conducted controlled experiments on rats age 24 d in order to compare the effect of gonadotropins and LR^3-IGF-1 separately or in combination *(53)*. LR^3-IGF-1 or saline (control) was infused by a continuous infusion pump for a period of 5 d (age 24–29 d). The dose of LR^3-IGF-1 was on the basis of previous dose-response studies that demonstrated biological efficacy *(53,54)*. At age 27 d, rats were randomly selected to receive either an sc injection of 15 IU of eCG or saline (control). The injection was given at 9:00 AM and the rats were sacrificed 48 h later, which was equivalent to proestrus for eCG-treated rats. In this manner, the four in vivo treatment groups were as follows: control, eCG, LR^3-IGF-1, and eCG + LR^3-IGF-1. OCCs and MGCs were obtained and cultured in vitro with four doses of FSH (0, 2, 10, and 50 mg/mL). In this manner, we were able to compare the four in vivo treatments of IGF-1 analog by itself or in combination with gonadotropins.

The dose-response curve to FSH (in vitro) with prior treatment with eCG was evaluated (Figs. 12 and 13). It showed that previous exposure to eCG increased the rate of ^{3}H-thymidine incorporation at all dose levels of FSH in MGCs and most dose levels of FSH in CCs. The shape of the response curve was different between CCs and MGCs. The dose-response curve to FSH with prior treatment with LR^3-IGF-1 was also studied. It showed that prior treatment with IGF-1 (analog) does not appear to affect subsequent cell replication. A dose-response curve to FSH with prior exposure to a combination of gonadotropin and IGF-1 (analog) was evaluated. This combination appeared to have a more modest effect on cell replication as compared to prior exposure to gonadotropin alone.

Previous studies have focused on FSH and IGF-1 interactions in MGCs. An experiment was designed to study these interactions in CC proliferation *(56)*. Immature rats received one of the following in vivo treatments: saline, eCG with high FSH activity, an IGF-1 analog (LR^3-IGF-1) with poor binding to IGF-1 binding proteins, or a combination of both hormones. CCs from each in vivo treatment were then cultured and treated in vitro with either saline, FSH, or IGF-1. CC proliferation was assessed by measurement of ^{3}H-thymidine incorporation; the results are shown

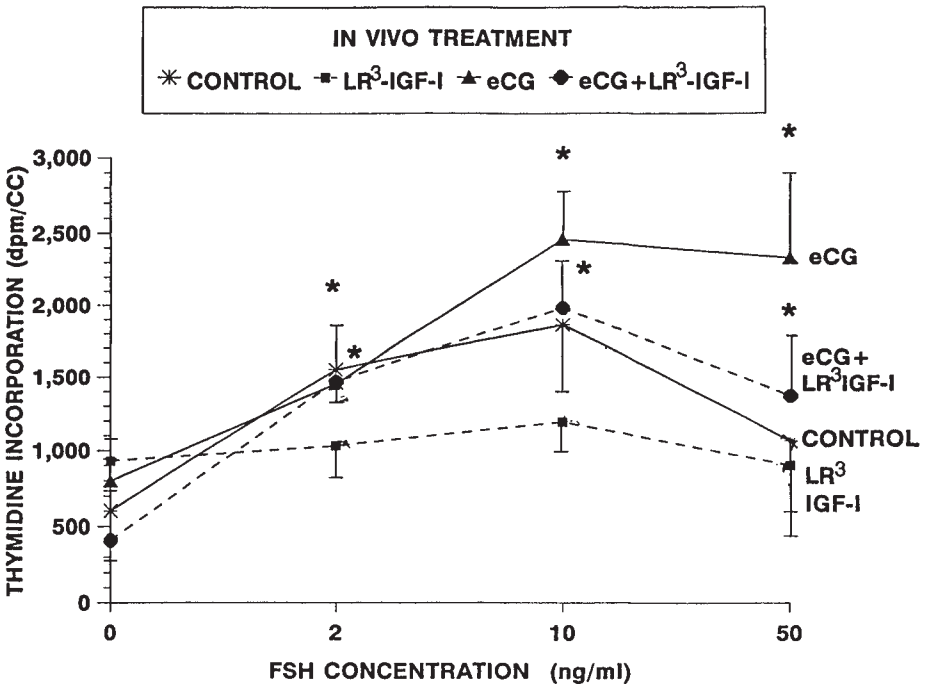

Fig. 12. ^{3}H-Thymidine incorporation of OCCs from rats treated in vivo with eCG, LR^{3}-IGF-1, both, or saline. Cultured OCCs were treated in vitro with different concentrations of FSH. Mean values of ^{3}H-thymidine incorporation are reported per each OCC. Data are mean values ± SEM for pooled data from two experiments, with six in vitro replicates per FSH concentration. Dashed lines represent groups that received LR^{3}-IGF-1 (either LR^{3}-IGF-1 or eCG + LR^{3}-IGF-1). Asterisks indicate values significantly different from control (no IGF-1), within the same in vivo treatment ($p < 0.05$). [Reprinted by permission of the Society of Endocrinology (*Endocrinology* 2001; **170,** 565–573).]

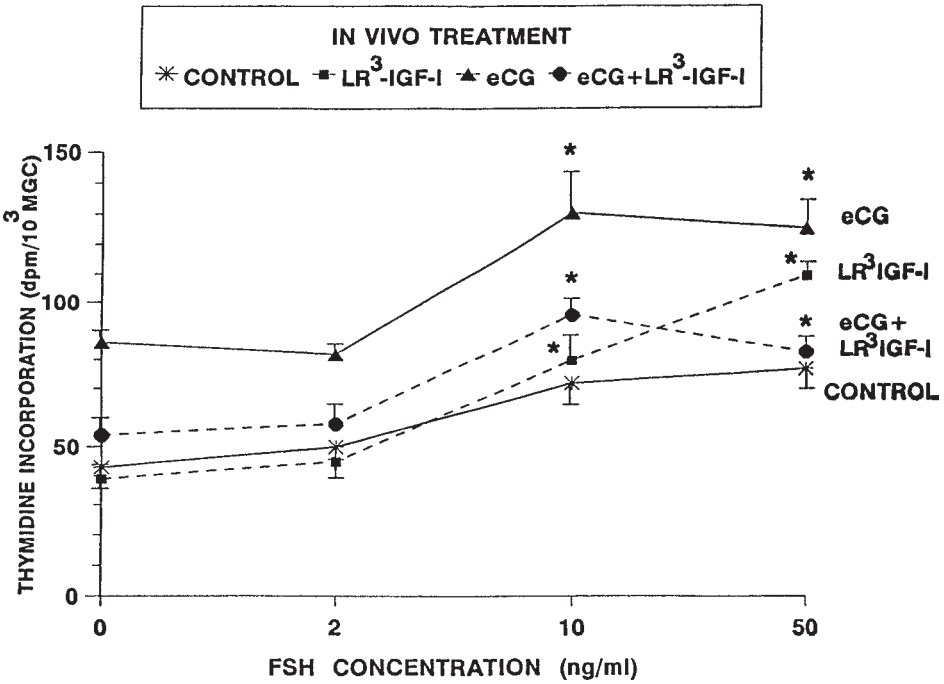

Fig. 13. ^{3}H-Thymidine incorporation of MGCs from rats treated in vivo with eCG, LR^{3}-IGF-1, both, or saline. Cultured MGCs were treated in vitro with different concentrations of FSH. Mean values of ^{3}H-thymidine incorporation are reported for 10^{3} MGCs. Data are mean values ± SEM for pooled data from two experiments, with six in vitro replicates per FSH concentration. Dashed lines represent groups that received LR^{3}-IGF-1 (either LR^{3}-IGF-1 or eCG + LR^{3}-IGF-1). Asterisks indicate values significantly different from control (no IGF-1), within the same in vivo treatment ($p < 0.05$). [Reprinted by permission of the Society of Endocrinology (*Endocrinology* 2001; **170,** 565–573).]

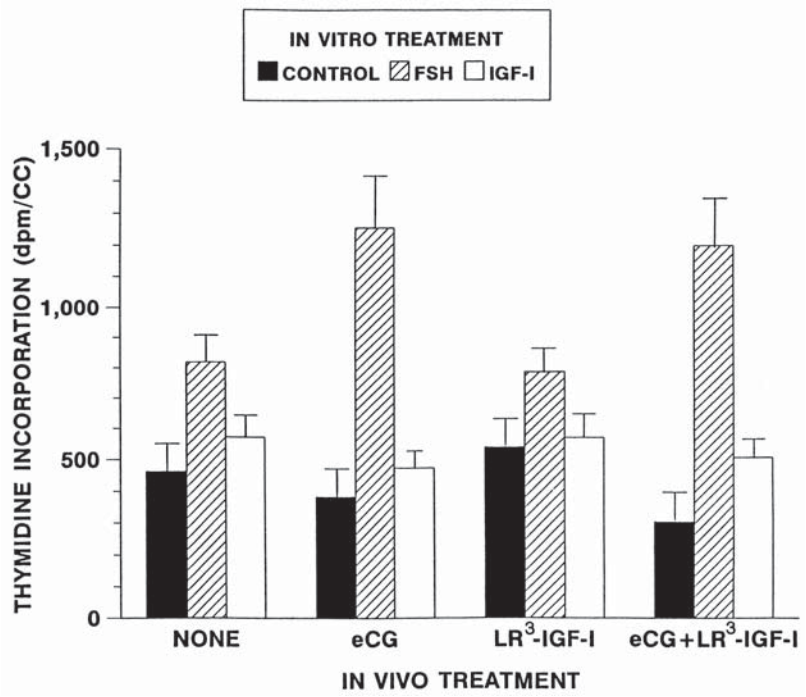

Fig. 14. ^{3}H-Thymidine incorporation of oocyte-cumulus complexes (CC) from rats treated in vivo with eCG, an IGF-1 analog (LR^3-IGF-1), both, or saline. Cultured CCs were treated in vitro with either FSH (5 ng/mL), IGF-1 (10 ng/mL), or saline before being challenged with ^{3}H-thymidine for measurement of replication. Mean values of ^{3}H-thymidine incorporation are reported per each CC. Treatment with FSH in vitro increased ^{3}H-thymidine incorporation over all in vivo treatments ($p < 0.05$). Treatment with IGF-1 in vitro increased DNA synthesis in eCG + LR^3-IGF-1-treated rats ($p < 0.01$). (Reprinted by permission from *Endocrine* 2000; **12,** 41–45.)

in Fig. 14. Prior in vivo treatment with eCG resulted in the highest proliferative activity of CCs when combined with FSH in vitro treatment. In vivo treatment with LR^3-IGF-1 had no effect on CC replication. CC replication was higher in FSH in vitro treatment than in IGF-1. The combination of eCG and LR^3-IGF-1 was the only in vivo treatment to stimulate higher CC proliferation with IGF-1 in vitro treatment. This study suggests that FSH does not act through IGF-1, a mechanism previously proposed.

Effect of Igf-1 on Folliculogenesis

When IGF-1 is administered parenterally, its action is modulated in different ways by several binding proteins. The development of an analog of IGF-1 (LR^3-IGF-1), which binds very poorly to IGF-1-binding proteins, allowed evaluation of physiology/pharmacology of IGF-1 without the modulating influence of the binding proteins *(57)*. In this manner, several parameters of the influence of IGF-1 (analog) on the ovarian function were assessed, which are discussed next.

Ovarian Weight

Three different strains of rats that were at different stages of maturity at d 24 of age were studied. The effects of saline, LR^3-IGF-1, eCG, and a combination of LR^3-IGF and eCG were studied on the body weight and ovarian weight (Fig. 15). No effect was noted on the body weight, but in all three strains LR^3-IGF-1 increased the ovarian weight in the groups treated with eCG. This reached statistical significance in two strains (Dark Agouti and Long Evans).

Ovulation Rate

Ovulation rate is defined as the number of oocytes released from the ovaries. Table 7 demonstrates the experimental design when ovulation rate was compared among four in vivo treatments: (A) saline alone, (B) LR^3-IGF alone, (C) saline plus eCG, and (D) combination of LR^3-IGF-1 and eCG; Figure 16 presents the results. LR^3-IGF-1 did not increase the ovulation rate, whereas eCG did have a significant effect on the ovulation rate. The addition of LR^3-IGF-1 to eCG further increased the ovulation rate (beyond what could be achieved by eCG alone).

Cumulus Cells

Figure 17 shows the number of CCs per each OCC. Note that eCG or IGF-1, significantly reduced the number of CCs per OCC. Past research has demonstrated that when ovaries are hyperstimulated with gonadotropins, the size of the OCC will be directly related to fertilization and early embryonic development *(58)*. Increased ovulation rate with eCG, LR^3-IGF-1, or their combination is related to reduced number of CCs per each OCC. Therefore, the increase in the number of oocytes may not necessarily lead to an equivalent increase in the number of good embryos.

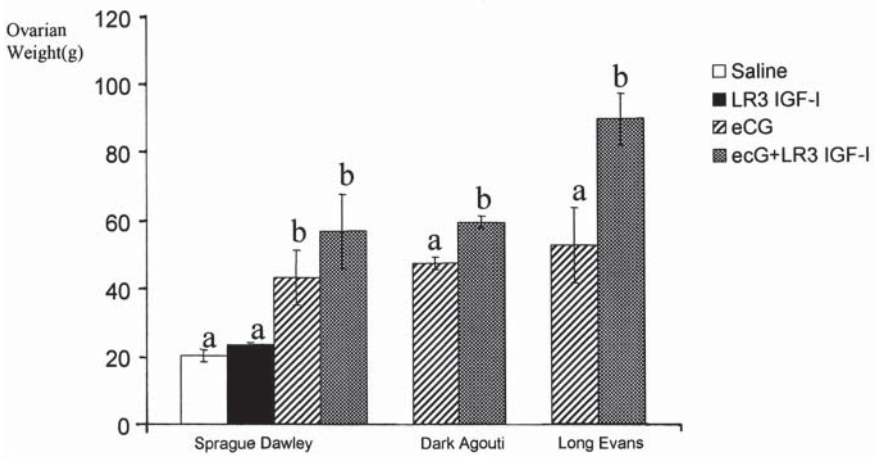

Fig. 15. Effect of saline, LR^3-IGF-1, eCG, or LR^3-IGF-1 on ovarian weight of three strains of rats that mature at a different rate. Different letters indicate significant difference at $p < 0.05$. (Reprinted by permission from *Endocrine* 2001; **14,** 175–180.)

Table 7
Experimental Design[a]

	Treatment subgroup			
	A	B	C	D
Number of rats	14	14	14	14
Infusion d 24–30	Saline	LR^3-IGF-1	Saline	LR^3-IGF-1
Injection on d 27	Saline	Saline	eCG	eCG
Injection on d 29	hCG	hCG	hCG	hCG
Rat sacrifice on d 30	All	All	All	All

[a]Reprinted by permission from *Endocrine* 2001; **14,** 175–180.

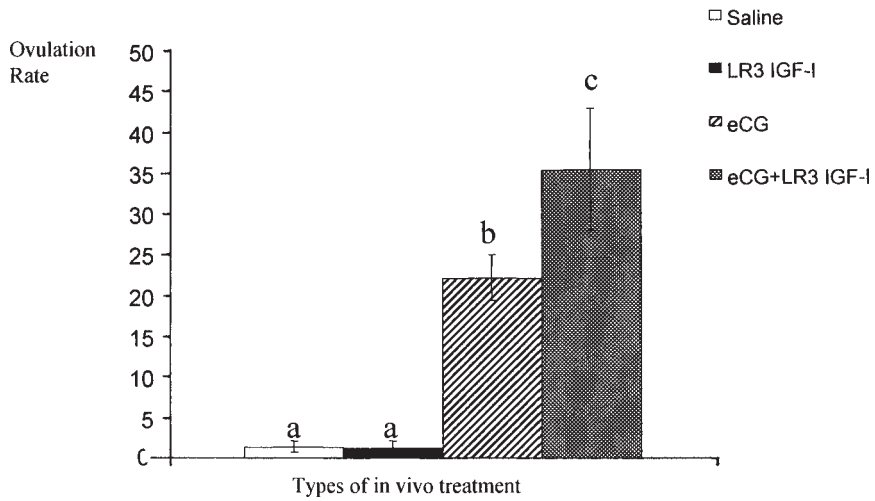

Fig. 16. Correlation of type of in vivo treatment with ovulation rate demonstrated in Sprague-Dawley rats. The different letters indicate statistical difference of $p < 0.05$. (Reprinted by permission from *Endocrine* 2001; **12,** 175–180.)

Recent research has clarified the physiological role and possible pharmacological utilization of IGF-1. Other growth factors have entered the realm of medical treatment, and it is possible to speculate about clinical use of IGF-1 for increased ovulation.

Conclusion

On the basis of the studies reviewed the following conclusions can be made:

1. *Action of IGF-1 on cell replication:* It is now clear that IGF-1 has a very important and, probably, an obligatory role in granulosa cell replication in all species tested. The reason that previously this effect was not seen in the rat model is that granulosa cells require exposure to FSH prior to being able to replicate under the influence of IGF-1 *(52)*.
2. *FSH and IGF-1 interact in a variety of ways:*
   a. *Obligatory action:* When rat granulosa cells were not exposed to FSH, they did not replicate *(52)*. Mice defi-

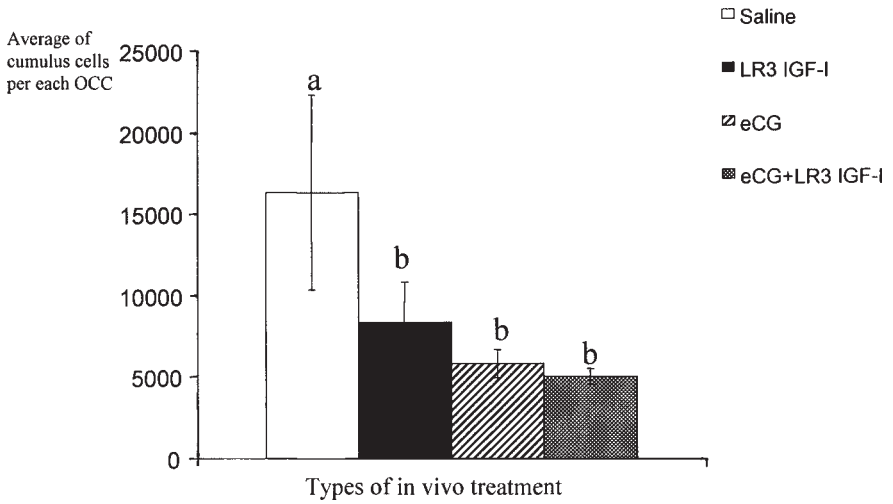

Fig.17. Correlation of type of in vivo treatment with number of same size CCs per each OCC in Sprague-Dawley rats. The different letters signify statistically significant difference. (Data are from ref. *57*.)

cient in IGF-1 gene did not ovulate in response to FSH *(45)*.

b. *Synergistic action:* In certain situations a combination of FSH and IGF-1 is more effective than the mere mathematical addition of the two effects. This was repeatedly demonstrated pertaining to progesterone secretion *(50)*.
c. *Additive action:* In certain circumstances, FSH and IGF-1 act merely additively. This was demonstrated pertaining to DNA synthesis in bovine granulosa cells *(50)*.
d. *Antagonistic action:* In granulosa cells of the cumulus oophorus in the bovine model, IGF-1 caused a significant increase in DNA synthesis. The addition of FSH blocked this effect *(51)*. The mechanism of this was shown to be owing to differentiation of CCs, making them responsive to the proliferative effect of IGF-1.

3. *There are two distinct types of granulosa cells related to their proximity to the oocyte:* These two types respond differently to FSH, IGF-1, and various combinations of the latter. These changes may well be related to the influence of the oocyte on its surrounding cells *(52,55,56)*. In future research, this point will have to be seriously considered. Cumulus oophorus is emerging to be a very important factor in oocyte maturation *(58)*.
4. *Combination of IGF-1 with FSH improves ovulation rate:* This will have to be further explored since analogs of IGF-1 may have a beneficial effect in the induction of superovulation in women who are going through infertility treatment *(57)*. In farm animals, this may lead to efficiency in production of more protein to feed an ever-increasing world population.

Acknowledgments

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References

1. Dorrington, J. H. and Armstrong, D. T. (1979). *Recent Prog. Horm. Res.* **35**, 301–342.
2. Richards, J. S. (1980). *Physiol. Rev.* **60,** 51–89.
3. Savion, N., Lui, G.-M., Laherty, R., and Gospodarowicz, D. (1981). *Endocrinology* **109,** 409–417.
4. May, J. V., Frost, J. P., and Schomberg, D. W. (1988). *Endocrinology* **123,** 168–179.
5. Greenwald, G. S. and Terranova, P. F. (1988). In: *The physiology of reproduction*, vol. 1. Knobil, E. and Neill, J. (eds.). Raven: New York.
6. Adashi, E. Y., Resnick, C. E., D'ercole, A. J., et al. (1985). *Endocr. Rev.* **6(3),** 400–420.
7. Singh, B. and Armstrong, D. T. (1994). *Theriogenology* **41,** 295–301.
8. Erickson, G. F. and Shimasaki, S. (1996). Department of Reproductive Medicine (0674), University of California.
9. Murphy, L. J., Bell, G. I., and Friesen, H. G. (1987). *Endocrinology* **120,** 1279–1282.
10. Oliver, J. E., Aitman, T. J., Powell, J. F., Wilson, C. A., and Ciayton, R. N. (1989). *Endocrinology* **124,** 2670–2679.
11. Zhou, J., Chin, E., and Bondy, C. (1991). *Endocrinology* **129,** 3281–3288.
12. Hernandez, E. R., Roberts, C. H., LeRoith, D., and Adashi, E. Y. (1989). *Endocrinology* **125,** 572–574.
13. Hansson, H.-A., Nilsson, A., Isgaard, J., Billig, H., Isaksson, O., Skottner, A., Andersson, I. K., and Rozell, B. (1988). *Histochemistry* **89,** 403–410.
14. Goldenberg, R. L., Vaitukaitis, J. L., and Ross, G. T. (1972). *Endocrinology* **90,** 1492–1498.
15. Lonergan, P., Monaghan, P., Rizos, D., Boland, M. P., and Gordon, I. (1994). *Mol. Reprod. Dev.* **37,** 48–53.
16. Hsu, C.-J. and Hammond, J. M. (1986). *Endocrinology* **120,** 198–207.
17. Hammond, J. M., Hsu, C.-J., Klindt, J., Tsang, B. K., and Downey, B. R. (1988). *Biol. Reprod.* **38,** 304–308.

18. Magoffin, D. A. and Erickson, G. F. (1988). *Cell. Dev. Biol.* **24,** 862–870.
19. Magoffin, D. A., Kurtz, K. M., and Erickson, G. F. (1990). *Mol. Endocrinol.* **4,** 489–496.
20. Hernandez, E. R., Resnick, C. E., Svoboda, M. E., Van Wyk, J. J., Payne, D. W., and Adashi, E. Y. (1988). *Endocrinology* **122,** 1603–1612.
21. Caubo, B., De Vinna, R. S., and Tonetta, S. A. (1989). *Endocrinology* **125,** 321–326.
22. Cara, J. F. and Rosenfield, R. L. (1988). *Endocrinology* **123,** 733–739.
23. Cara, J. F., Fan, J., Azzarello, J., and Rosenfield, R. L. (1990). *J. Clin. Invest.* **86,** 560–565.
24. Magoffin, D. A. and Weitsman, S. R. (1994). *Biol. Reprod.* 1994; **51,** 766–775.
25. Magoffin, D. A. and Weitsman, S. R. (1993). *Biol. Reprod.* **48,** 1166–1173.
26. Magoffin, D. A. and Weitsman, S. R. (1993). *Mol. Cell. Endocrinol.* **96,** 45–51.
27. Magoffin, D. A. and Weitsman, S. R. (1993). *Endocrinology* **132,** 1945–1951.
28. Nakatani, A., Shimasaki, S., Erickson, G. F., and Ling, N. (1991). *Endocrinology* **129,** 1521–1529.
29. Erickson, G. F., Nakatani, A., Ling, N., and Shimasaki, S. (1992). *Endocrinology* **130,** 625–636.
30. Erickson, G., Nakatani, A., Ling, N., and Shimasaki, S. (1992). *Endocrinology* **130,** 1867–1878.
31. Adashi, E. Y., Resnick, C. E., Svoboda, M. E., and Van Wyk, J. J. (1986). *Biol. Reprod.* **34,** 81–88.
32. Ricciarelli, E., Hernandez, E. R., Hurwitz, A., Kokia, E., Rosenfeld, R. G., Schwander, J., and Adashi, E. Y. (1991). *Endocrinology* **129,** 2266–2268.
33. Ricciarelli, E., Hernandez, E. R., Kokia, E., Rohan, R. M., Tedeschi, C., Botero, L., Rosenfeld, R. G., Albiston, A. L., Herington, A. C., and Adashi, E. Y. (1992). *Endocrinology* **130,** 3092–3094.
34. Rohan, R. M., Ricciarelli, E., Kiefer, M. C., Resnick, C. E., and Adashi, E. Y. (1993). *Endocrinology* **132,** 2507–2512.
35. Ui, M., Shimonaka, M., Shimasaki, S., and Ling, N. (1989). *Endocrinology* **25,** 912–916.
36. Shimasaki, S., Shimonaka, M., Ui, M., Inouye, S., Shibata, F., and Ling, N. (1990). *J. Biol. Chem.* **265,** 2198–2202.
37. Bicsak, T. S., Shimonaka, M., Malkowski, M., and Ling, N. (1990). *Endocrinology* **126,** 2184–2189.
38. Bicsak, T. S., Long, N., and DePaolo, L. V. (1991). *Biol. Reprod.* **44,** 599–603.
39. Shimasaki, S., Murakami, K., Morita, Y., and Erickson, G. F. (1995). *Frontiers in endocrinology*. Serono Symposium, Elsevier, New York.
40. Erickson, G. F., Nakatani, A., Liu, X.-J., Shimasaki, S., and Ling, N. (1994). In: *Molecular biology of the female reproductive system*. Findlay, J. K. (ed.). Academic: New York.
41. Liu, X.-J., Malkowski, M., Guo, T.-L., Erickson, G. F., Shimasaki, S., and Ling, N. (1993). *Endocrinology* **132,** 1176–1183.
42. Erickson, G. F., Li, D., Sadkhanloo, R., Liu, X.-J., Shimasaki, S., and Ling, N. (1994). *Endocrinology* **134,** 1365–1372.
43. Erickson, G. F., Li, D., Weitsman, S. R., Shimasaki, S., Ling, N., and Magoffin, D. A. (1995). *Endocrine* **3,** 525–531.
44. Yoshimura, Y., Aoki, N., Sueoka, K., Miyazarki, T., Kuji, N., Tanaka, M., and Kobayashi, T. J. (1996). *Clin. Invest.* **98(2),** 308–316.
45. Efstratiadis, A. (1994). In: *Developmental endocrinology, frontiers in endocrinology*, vol. 6. Sizonenko, P. C., Aubert, M. L., and Vassali, J. D. (eds.). Ares-Serono Symposia Publications.
46. Xia, P., Tekpetey, F. R., and Armstrong, D. T. (1994). *Mol. Reprod. Dev.* **38,** 373–379.
47. Brower, P. T. and Schultz, R. M. (1982). *Dev. Biol.* **90,** 144–153.
48. Moore, R. M. (1983). In: *Current problems in germ cell differentiation*. McLaren, A. and Wylie, C. C. (eds.). University Press: Cambridge, UK.
49. Adashi, E. Y., Resnick, C. E., Dercole, A. J., Svoboda, M. C., and Van Wyk, J. J. (1985). *Endocr. Rev.* **6(3),** 400–420.
50. Armstrong, D. T., Xia, P., de Gannes, G., Tekpetey, F. R., and Khamsi, F. (1996). *Biol. Reprod.* **54,** 331–338.
51. Khamsi, F. and Armstrong, D. T. (1997). *Biol. Reprod.* **57,** 684–688.
52. Khamsi, F. and Roberge, S. (2001). *Fertil. Steril.* **75,** 997–1002.
53. Khamsi, F. and Roberge, S. (2001). *J. Endocrinol.* **170,** 565–573.
54. Francis, A. L., Ross, M., Ballard, F. J., Milner, S. J., Senn, E., McNeil, K. M., Wallace, J. C., King, R., and Wells, J. R. E. (1992). *J. Mol. Endocrinol.* **8,** 213–230.
55. Ballard, F. J., Francis, A. L., Walton, P. E., Knowles, S. E., Owens, P. C., Read, L. C., and Tomas, F. M. (1993). *Aust. J. Agric. Sci.* **44,** 567–577.
56. Khamsi, F. and Roberge, S. (2000). *Endocrine* **12(1),** 41–45.
57. Khamsi, F., Roberge, S., and Wong, J. (2001). *Endocrine* **14(3),** 175–180.
58. Khamsi, F., Roberge, S., Lacanna, I., Wong, J., and Yavas, Y. (1999). *Endocrine* **10,** 161–166.