Molecular Characterization and Regulation of Gonadotropin-Releasing Hormone (GnRH) and its Receptor mRNA in the Human Ovary

By

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ABSTRACT

With the recent detection of GnRH and its receptor (GnRH-R) in numerous extrapituitary tissues, it is hypothesized that GnRH acts as an autocrine/paracrine regulator of ovarian function. The direct involvement of GnRH in follicular atresia and modulation of ovarian steroidogenesis, further substantiate the extrapituitary actions of this decapeptide. In contrast to the hypothalamus and pituitary, the factors that regulate ovarian GnRH and its receptor remain poorly characterized. As gonadal steroids are key regulators of ovarian functions, the present study investigated the role of 17β-estradiol (E2) and progesterone (P4) in regulating the intrinsic ovarian GnRH axis. Using reverse transcription polymerase chain reaction (RT-PCR), we have isolated the full-length GnRH-R coding region from human granulosa-luteal cells (hGLCs). Sequence analysis revealed that the ovarian GnRH-R cDNA is identical to its pituitary counterpart. Basal expression studies demonstrated that GnRH and GnRH-R mRNA levels significantly increased with time in culture, reaching levels of 160% and 170% on day 8 and 10 of culture compared to day 1 ($p<0.05$), respectively. On day 5 in culture, hGLCs were treated with E2 and RU486 (a progesterone receptor antagonist) in a dose- and time-dependent fashion. A dose-dependent decrease was observed in GnRH and GnRH-R mRNA levels after a 24h treatment with E2 (1-100 nM). Time course studies demonstrated that E2 (1 nM) decreased GnRH mRNA levels in a time dependent manner, with a maximal inhibition of 40% at 48h ($p<0.05$). In contrast, GnRH-R expression exhibited a biphasic pattern with time. A 6h treatment with E2 (1 nM) resulted in a 20% increase in GnRH-R message ($p<0.05$), whereas a long-term treatment (48h) resulted in a 60% decrease in GnRH-R expression ($p<0.05$) in hGLCs. Tamoxifen treatment reversed

the E2-induced inhibition of GnRH and GnRH-R mRNA expression, indicating that the E2 effect was mediated through its receptor. The progesterone receptor antagonist had no signficant effect on GnRH mRNA levels in hGLCs. However, RU486 induced a time- and dose-dependent decrease in GnRH-R mRNA expression, with a maximal inhibition of 50% at a dose of 10μM for 24h ($p<0.05$). Since GnRH actions have been associated with atresia and luteolysis, the dynamic balance between E2 and P4 may contribute to the maintenance of the corpus luteum.

With the recent identification of a second form of GnRH in the human brain (GnRH-II), the present study examined the expression and function of GnRH-II in human ovarian cells. Using RT-PCR we demonstrated the expression of GnRH-II in hGLCs, an ovarian cancer cell line (OVCAR-3), primary ovarian cancer cells, and human ovarian surface epithelial cells. Functionally like GnRH-I, GnRH-II significantly decreased progesterone secretion in hGLCs ($p<0.001$). Furthermore, GnRH-II decreased progesterone secretion more than GnRH-I (50% versus 30% compared to control; $p<0.05$). The GnRH-induced inhibition of progesterone secretion was reversed by antide, a GnRH antagonist.

In summary, our studies demonstrate that the ovary possesses an intrinsic GnRH axis that is dynamically regulated during spontaneous luteinization *in vitro*, and that gonadal steroids are capable of regulating GnRH and its receptor in the human ovary. Coupled with the expression and functional analysis of a second form of GnRH (GnRH-II) in the ovary, our studies strengthen the notion that GnRH acts as an autocrine/paracrine modulator of ovarian functions.

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LIST OF ABBREVIATIONS

ART	Assisted Reproductive Technologies
bp	Base pairs
C	Celcius
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucelic acid
cGnRH-II	Chicken II gonadotropin-releasing hormone
dNTP	deoxynucleoside triphosphate
FBS	Foetal bovine serum
E2	17β-estradiol
ER α/β	Estrogen Receptor α/β
ERE	Estrogen response element
Fas L	Fas Ligand
FSH	Follicle stimulating hormone
GAP	GnRH-associated peptide
GnRH	Gonadotropin-releasing hormone
GnRHa	Gonadotropin-releasing hormone agonist
GnRH-R	Gonadotropin-releasing hormone receptor
G-protein	GTP-binding protein
h	Hour
HBSS	Hanks balanced salt solution
hCG	Human chorionic gonadotropin

hGLCs	Human granulosa-luteal cells
IU	International unit
IVF	*In vitro* Fertilization
Kda	Kilodaltons
LH	Luteinizing hormone
μ	Micro
M199	Medium 199
MAP Kinase	Mitogen activated protein kinase
mGnRH	Mammalian gonadotropin-releasing hormone
ml	Mililiters
min	Minutes
MMP	Matrix metalloproteinases
mRNA	Messenger ribonucleic acid
n (as in nM)	Nano
p (as in pM)	Pico
P4	Progesterone
PBS	Phosphate buffered saline
PBS-G	Phosphate buffered saline-gelatin
PCR	Polymerase chain reaction
PGF2α	Prostaglandin F2α
PKC	Protein kinase C
PR	Progesterone receptor
RIA	Radioimmunoassay

RT-PCR	Reverse transcription polymerase chain reaction
s	Seconds
SE	Standard Error
TIMP	Tissue inhibitor of metalloproteinase
Txf	Tamoxifen

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A. BACKGROUND

I. Introduction

In humans, reproduction is controlled by the concerted regulation of the hypothalamic-pituitary-gonadal-axis. During the normal menstrual cycle, the dynamic feedback interactions of hormones and peptides from all levels of the axis tightly regulate sexual maturation and reproductive functions. Gonadotropin-releasing hormone (GnRH), a highly conserved hypothalamic decapeptide, plays an integral role in regulating reproduction. Secreted in a pulsatile manner into the hypothalmo-hypophyseal portal system (Noar et al., 1995), GnRH travels to the anterior pituitary gland and stimulates the synthesis and secretion of gonadotropins (Bauer-Danton and Jameson, 1995; Kaiser et al., 1997). The actions of GnRH are mediated through a high affinity, G-protein coupled receptor (GnRH-R) on the surface of the pituitary gonadotropes (Leung and Steele, 1992; Conn 1994). Synthesis and secretion of GnRH is modulated by a number of factors including autoregulation, catecholaminergic innervation, gonadotropin short feedback loops and gonadal steroids (Figure 1) (Sawyer, 1975; Ortmann et al., 1995; Valenca et al., 1987). Furthermore, GnRH actions may be modulated by factors that regulate its receptor transcription, translation and membrane targeting.

In addition to the hypothalamic-pituitary axis, GnRH and its receptor (GnRH-R) have been detected in other reproductive tissues including the gonads and placenta (Lin et al., 1995; Yin H et al.; Peng et al., 1994; Olofsson et al., 1995; Seeburg and Adelman, 1984; Tan and Rousseau, 1982). Although the functional role of the intrinsic GnRH axis in these tissues is poorly understood, the detection of the GnRH-R and its ligand in these

tissues suggests that GnRH plays and autocrine/paracrine role in regulating reproductive functions.

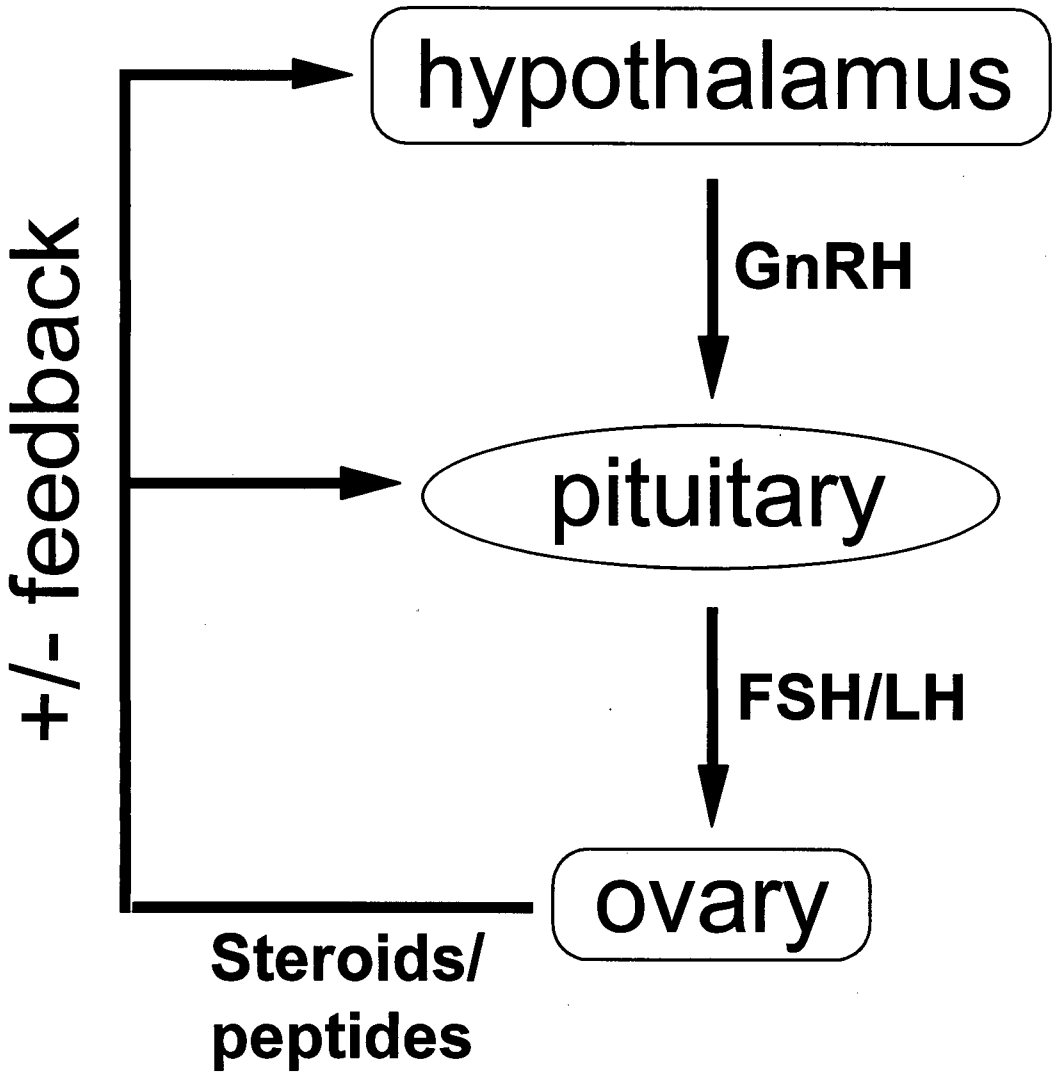


Figure 1. The hypothalamo-pituitary-gonadal axis. GnRH, a hypothalamic decapeptide, stimulates the synthesis and secretion of gonadotropins (FSH and LH) from the anterior pituitary gland. In the ovary, the gonadotropins stimulate follicular development and steroidogenesis. Intraovarian factors such as steroids and gonadal peptides, feedback to the hypothalamus and pituitary to tightly regulate the reproductive axis.

II. Pituitary Gonadotropins-Structure and Function

Activation of the GnRH-R by its cognate ligand results in phosphoinositide turnover, which stimulates release of intracellular Ca^{2+} and activation of protein kinase C (Hazum and Conn, 1988; Leung and Steele, 1992). This ultimately leads to the release of FSH and LH from the pituitary gonadotropes (Huckle and Conn 1988; Leung and Steele, 1992; Saunders et al., 1998).

The pituitary gonadotropins, LH and FSH, are classified as glycoprotein hormones which exist as a noncovalently associated heterodimer (Wierman et al., 1988). The α subunit is conserved amongst all glycoprotein hormones within species and is transcribed from a gene that is distinct from the β-subunit. The β subunit is responsible for the specificity of the hormone and association of the two subunits is required for function (Ryan et al., 1987). After synthesis is complete, the peptides undergo post-translational modifications, including N- or O-linked glycosylation (Pierce and Parsons, 1981; Gharib et al., 1990)

After secretion into the circulation, FSH and LH travel to the gonads and modulate gametogenesis and steroidogenesis. In males, LH stimulates testosterone production by the interstital cells of the testes, while FSH is involved in the upregulation of LH receptors on Leydig cells and stimulation of spermatogenesis. In females, LH induces ovulation and corpus luteum formation. In contrast, FSH is responsible for early development of the follicles that is accompanied by estrogen production in females. Molecular techniques have confirmed that FSH binds exclusively to granulosa cells, and this binding has been observed as early as the primary follicle stage (Gougeon, 1993). In

contrast, the effects of LH are not apparent until later in follicular development, as evident by the absence of LH receptors on granulosa cells early in the follicular phase. The gonadotropic effects are mediated through a G-protein coupled receptor that activates the adenylate cyclase effector system (Hsueh et al., 1984; Simoni et al., 1997). Recently, both of these receptors have also been linked to mobilization of intracellular calcium (Leung and Steele, 1992). Functionally, LH and FSH stimulate steroid biosynthesis by induction of steroidogenic enzymes, and their effects are regulated by GnRH and gonadal steroids (Hseuh et al., 1980, Hillier et al., 1995).

III. Gonadal Steroids and their Receptors

Derived from cholesterol, the major female sex steroids are 17β-estradiol (E2) and progesterone (P4). Their effects are mediated through cytoplasmic receptors that translocate to the nucleus and modulate gene transcription (Beato and Sanchez-Pacheco, 1996). Structurally, steroid receptors are composed of functional domains designated A to F. Upon binding steroids, the receptors dimerize and translocate to the nucleus (Carson-Jurica et al., 1990; Luisi et al., 1994). Until recently, it was thought that there was only one estrogen receptor (ER), namely ERα. However, a second estrogen receptor, ERβ has been cloned from the rat (Kuiper et al., 1996), mouse (Tremblay et al., 1997) and human (Mosselman S et al., 1996) reproductive tissues, including the ovary and testis. In the ovary, immunohistochemistry studies demonstrated a differential distribution of the two forms of ERs. ERα was primarily expressed in the ovarian stroma and theca cells, while ERβ was localized to the granulosa cells (Sar and Welsch, 1999; Fitzpatrick et al., 1999; Rosenfeld et al, 1999). Furthermore, recent studies have documented the expression of both ERs in the human corpus luteum and hGLCs (Brandenberger et al., 1998; Misao et al., 1999). However, the functional role of the two distinct receptors in estrogen mediated responses is not well understood. The intraovarian role of estrogen is species- and stage-specific. In general, E2 synergizes with the effects of gonadotropins by augmenting cAMP accumulation, aromatase activity and FSH/LH receptor content during the follicular phase of the menstrual cycle (Hseuh et al., 1984). Although studies have implicated E2 in modulating steroidogenesis during the

luteal phase of the menstrual cycle (Holt and Schreiber, 1985; Endo et al., 1998; Wuttke et al., 1998), the role of estrogen during this phase is poorly understood.

Like the ER, the progesterone receptor (PR) has two forms (PR_A and PR_B). However, the two isoforms of the PR arise from different splice variants of the same gene (Horowitz, 1992). There are at least nine different mRNA transcripts ranging from 2.5-11.4 kilobases, and there is evidence to suggest a potential third isoform (PR_C) in the T47D human breast cancer cell line (Wei et al., 1990; Wei and Miner, 1994). In contrast to the "B" form of the PR, the "A" form is truncated at the N-terminal portion of the peptide. As a result, the different isoforms arise from functionally different promoters. In the ovary, transcripts for the progesterone receptor are present during the periovulatory period (Park and Mayo, 1991), suggesting that progesterone may play a role in the ovulatory process (Baranczuk and Fainstat, 1976; Kohda et al., 1980). Although, PR immunoreactivity has been detected in epithelial, stromal and luteal cells of the primate ovary (Press and Greene, 1988; Chandrasekher et al., 1994), the role of progesterone as an autocrine/paracrine modulator of ovarian functions is not well understood. Chaffikin et al (1993) have demonstrated that P4 inhibits proliferation and differentiation of granulosa cells. However, this effect is diminshed by cAMP or hCG administration, indicating that the appropriate homeostatic balance between steroids and gonadotropins is crucial for transition to the corpus luteum.

IV. Regulation of the Hypothalamic-pituitary-gonadal axis

In contrast to the intraovarian effects of steroids, the feedback loops at the hypothalamo-pituitary axis are well characterized. During the follicular phase of the menstrual cycle, FSH stimulates the recruitment of a cohort of follicles from the ovarian reserve. This leads to the proliferation and differentaiton of the granulosa and theca cells around the oocyte, culminating in the production of E2 from the developing follicle. During this period, the rising E2 levels negatively feedback to the hypothalamo-pituitary axis and decrease FSH secretion (McNeilly, 1988). The molecular basis of the E2-induced negative feedback is based upon decreasing the frequency and amplitude of GnRH secretion from the hypothalamus, and the downregulation of GnRH-R expression on the pituitary gonadotropes. Furthermore, gonadotropins also exert short feedback loops to inhibit hypothalamic GnRH secretion. During the latter part of the follicular phase, E2 exerts a postive feedback effect at the hypothalamus and pituitary. The E2-induced increase in GnRH frequency coupled with increased responsivness of gonadotropes to GnRH, results in the midcycle gonadotropin surge (Yen, 1977; Channing et al., 1980; Turgeon, 1980). This surge is responsible for ovulation and maturation of the follicle. The remaining granulosa and theca cells become invaded with blood vessels and become the corpus luteum. During the luteal phase, E2 and P4 secreted by the corpus luteum inhibit the frequency of GnRH secretion, resulting in decreased levels of gonadotropin secretion. If fertilization does not occur, the corpus luteum undergoes regression by mechanisms still poorly understood, but have been related to a decreased responsivness to LH (Duncan et al., 1998). The resulting decline in steroids from the

regressing corpus luteum releases the negative feedback loop, culminating in an increase in FSH secretion and the beginning of a new cycle.

V. Distribution of GnRH and its Receptor

GnRH is synthesized and secreted from the pre-optic area (POA) and the arcuate nucleus of the hypothalamus (Sherwood et al., 1993; Lescheid et al., 1997). In addition, GnRH has been detected in other areas of the central nervous system including the amygdala, midbrain and spinal cord (Lescheid et al., 1997). Similarly, autoradiographic studies revealed that the GnRH-R has been detected outside of the pituitary, in areas such as the hippocampus, subiculum and parts of the cortex (Millan et al., 1985).

Outside of the hypothalamic-pituitary axis, GnRH and its receptor have been detected in numerous reproductive tissues. For example, GnRH has been detected in the ovary and testis in rats and primates, including humans (Oikawa et al., 1990; Dong et al., 1993; Dong et al., 1996). In the ovary, GnRH has been localized to the granulosa cells of developing follicles and the corpus luteum (Clayton et al., 1992; Whitelaw et al., 1995). Using radiolabelling techniques low affinity and high capacity binding sites for GnRH have been detected in the granulosa and luteal cells (Clayton et al., 1979 Bramely et al., 1986). Using RT-PCR, the expression of the GnRH-R gene has been confirmed in rat and human ovaries (Peng et al., 1994; Minaretzis et al., 1995; Olofsson et al., 1995). In the male, Seritoli cells express GnRH (Bahk et al., 1995), while Leydig cells express its receptor (Bourne et al., 1980).

In addition to the ovary, placental GnRH has been detected in the cytotrophoblasts and syncytiotrophoblasts (Seppala et al., 1980; Miyake et al., 1982). Using radioimmunoassays, GnRH levels significantly increased during the first trimester of pregnancy followed by a leveling off during the remaining gestational peroid (Siler-

Khodr and Khodr, 1978). Like its cognate ligand, binding studies indicated that the GnRH-R is present in the placenta and dynamically regulated during pregnancy (Currie et al., 1981; Lin et al., 1995). Furthermore, binding studies indicate that the endometrium possesses GnRH binding sites similar to the ovary and placenta (Casan et al., 1998).

GnRH and its binding sites have also been identified in a number of cancers including ovarian (Irmer et al., 1995), breast (Harris et al., 1991), endometrial (Imai et al., 1994) and prostate cancers (Qayum et al., 1990).

VI. Extrapituitary actions of GnRH

As aformentioned, GnRH and its receptor have been detected in numerous extrapituitary tissues including, the gonads, placenta, endometrium and reproductive tumors. Although the functions of GnRH in these tissues is not well understood, GnRH has hypothesized to act as an autocrine/paracrine regulator of reproductive functions (Peng et al., 1994; Stojilkovic et al., 1994; Tsafriri and Adashi, 1994).

VI-1. Intraovarian GnRH

In the ovary, biological responses to activation of the GnRH-R by its ligand are dependent upon the stage of the menstrual cycle. During the follicular phase, GnRH inhibits gonadotropin-induced steroidogenesis and granulosa cell differentiation. It is well documented that GnRH-R expression is high in atretic rat follicles, providing strong evidence for a role of GnRH in atresia (Whitelaw et al., 1995). This idea is further corraborated by the direct involvement of GnRH-induced apoptosis in rat granulosa cells (Billing et al., 1994). During the periovulatory period, GnRH induced follicular rupture and oocyte maturation in rats. These effects may be mediated by the increase in GnRH-induced transcription of genes such as tissue plasminogen activator (Ny et al., 1987), prostoglandin endoperoxidase synthase II (Wong and Richards, 1992), and the progesterone receptor (Natraj and Richards, 1993).

During the luteal phase of the menstrual cycle, the role of GnRH is not well understood. There are reports to suggest that GnRH is actively involved in the process of luteinization and luteolysis. For example, GnRH stimulated the expression of matrix

metalloproteinase (MMP) type 1 and 2 (MMP-1, MMP-2) in the corpus luteum. These enzymes are involved in degrading collagens and remodelling the extracellular matrix, leading to structural luteolysis in rats (Goto et al., 1999). During the early stages of pregnancy in the rat, GnRH increased corpus luteum DNA fragmentation (Sridaran R et al., 1998). Similarly, GnRH increased the number of apoptotic nuclei in human granulosa cells obtained during oocyte retrieval for *in vitro* fertilization (Zhao et al., 2000). Furthermore, recent evidence indicates that rat ovarian GnRH mRNA levels and serum progesterone levels are inversely related during pregnancy and parturition (Sridaran R et al., 1999), thereby providing evidence that GnRH may regulate ovarian steroidogenesis. Indeed, a number of studies have demonstrated the effect of GnRH on steroidogenesis in human granulosa-luteal cells (hGLCs). We and others have reported that GnRH inhibits progesterone secretion from hGLCs (Tureck et al., 1982; Peng et al., 1994). However, some groups reported a stimulation of progesterone production with GnRH treatment in cultured human granulosa cells (Parinnaud J et al., 1988; Olsson et al., 1990). The variability may be due to the concentrations, time and culture conditions. Finally, GnRH may interact with gonadotropins to modulate ovarian steroidogenesis (Leung and Steele, 1992; Vaannanen et al., 1997). Thus, to date the effect of GnRH on steroidogenesis remains controversial.

VI-2. Placental and Endometrial GnRH

In the placenta, GnRH stimulates the synthesis and secretion of human chorionic gonadotropin (hCG). (Siler-Khodr et al., 1986; Wide et al., 1988; Currie et al., 1993). Using GnRH-R antagonists, studies confirmed that the GnRH-induced hCG biosynthesis is a receptor mediated event (Siler-Khodr et al., 1983; Siler Khodr et al., 1987). The co-

localization of the GnRH-R and its ligand in this endocrine organ suggest a possible autocrine/paracrine role for GnRH in the human placenta. Interestingly, GnRH and GnRH-R expression in the placenta is relatively high during the first trimester of pregnancy, followed by a decrease in the GnRH-R in the second trimester and term placenta. Recent evidence also suggests that GnRH enhances implantation during murine embryonic development (Raga et al., 1999). Since implantation is a dynamic process that requires trophoblast invasion and extensive tissue remodelling, the balance between matrix metalloproteinases (MMP) and their inhibitors (TIMPS) is crucial. Furthermore, GnRH decreased TIMP mRNA expression in human cultured stromal cells (Raga et al., 1999). These observations provide strong evidence that GnRH may play an important role in the implantation process by modulating expression of extracellular matrix remodelling proteins. The role of GnRH in implantation has also been demonstrated in vivo. Women undergoing IVF had higher pregnancy rates if they were administered GnRH analogues during the early perimplantation stages (Balasch et al., 1993; Gartner et al., 1997).

VI-3. Reproductive Tumors

GnRH and its receptor have been detected in ovarian, breast, endometrial and prostate cancers (Imai et al., 1994; Eidne et al., 1985; Gallagner et al., 1991; Bahk et al., 1998). *In vitro* studies have demonstrated that GnRH inhibits tumor cell growth and may induce apoptotic cell death (Dondi et al., 1994; Palyi et al., 1996; Szepeshazi et al., 1997). Furthermore, GnRHa have been used to treat specific reproductive cancers in a clinical setting.

VII. Structure of GnRH and its Receptor

Throughout evolution the gene for gonadotropin-releasing hormone has been duplicated and structurally re-organized. Hence, throughout the animal kingdom there are multiple forms of GnRH. To date, at least 13 distinct forms of GnRH have been isolated and characterized (Table 1) (Carolsfield et al., 2000). Interestingly, within the same species multiple forms of GnRH may co-exist and have differential tissue distribution (Montero M et al., 1995; Urbanski HF et al., 1999; Schalburg et al., 1999). Until recently, reproduction in humans was thought to be exclusively controlled by one form of GnRH, namely mammalian GnRH (mGnRH). However, a second form of GnRH analogous to chicken II GnRH (cGnRH-II), has been cloned from the human brain (White et al., 1998). Administration of cGnRH-II significantly increased plasma LH levels during luteal phase in the rhesus monkey, indicating that the second form of GnRH is biologically active in the mammal (Lescheid et al., 1997).

Structurally, the genes that encode for the different forms of GnRH are very similar, thereby reinforcing a common genetic ancestry (Figure 2). The genes consists of four exons and three introns (Adelman et al., 1986; Sherwood et al., 1993). In humans, the gene for GnRH-I is located on chromosome 8, whereas the gene for GnRH-II is located on chromosome 20 (Hseuh et al., 1983; White et al., 1998). The gene for GnRH-II is much smaller (2.1 kb) than GnRH-I (5.1 kb) (Hayflick et al., 1989). However, the structure of the mRNA transcript from the gene is very similar. GnRH is derived from a preprohormone which consists of a signal peptide, and a GnRH associate peptide (GAP). After translation, this preprohormone undergoes post-translational modifications to yield

Table 1: Primary Amino Acid Sequences of known GnRH Structures

GnRH	***1 2 3 4 5 6 7 8 9 10***
Mammal	p-Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2
Catfish	p-Glu-His-Trp-Ser-His-Gly-Leu-Asn-Pro-Gly-NH_2
Seabream	p-Glu-His-Trp-Ser-Tyr-Gly-Leu-Ser-Pro-Gly-NH_2
Chicken I	p-Glu-His-Trp-Ser-Tyr-Gly-Leu-Gln-Pro-Gly-NH_2
Chicken II	p-Glu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH_2
Dogfish	p-Glu-His-Trp-Ser-His-Gly-Trp-Leu-Pro-Gly-NH_2
Salmon	p-Glu-His-Trp-Ser-Tyr-Gly-Trp-Leu-Pro-Gly-NH_2
Guinea pig	p-Glu-Tyr-Trp-Ser-Tyr-Gly-Val-Arg-Pro-Gly-NH_2
Tunicate I	p-Glu-His-Trp-Ser-Asp-Tyr-Phe-Lys-Pro-Gly-NH_2
Tunicate II	p-Glu-His-Trp-Ser-Leu-Cys-His-Ala-Pro-Gly-NH_2
Lamprey I	p-Glu-His-Tyr-Ser-Leu-Glu-Trp-Lys-Pro-Gly-NH_2
Lamprey III	p-Glu-His-Trp-Ser-His-Asp-Trp-Lys-Pro-Gly-NH_2
Herring	p-Glu-His-Trp-Ser-His-Gly-Leu-Ser-Pro-Gly-NH_2

Adapted from Carolsfeld et al., 2000

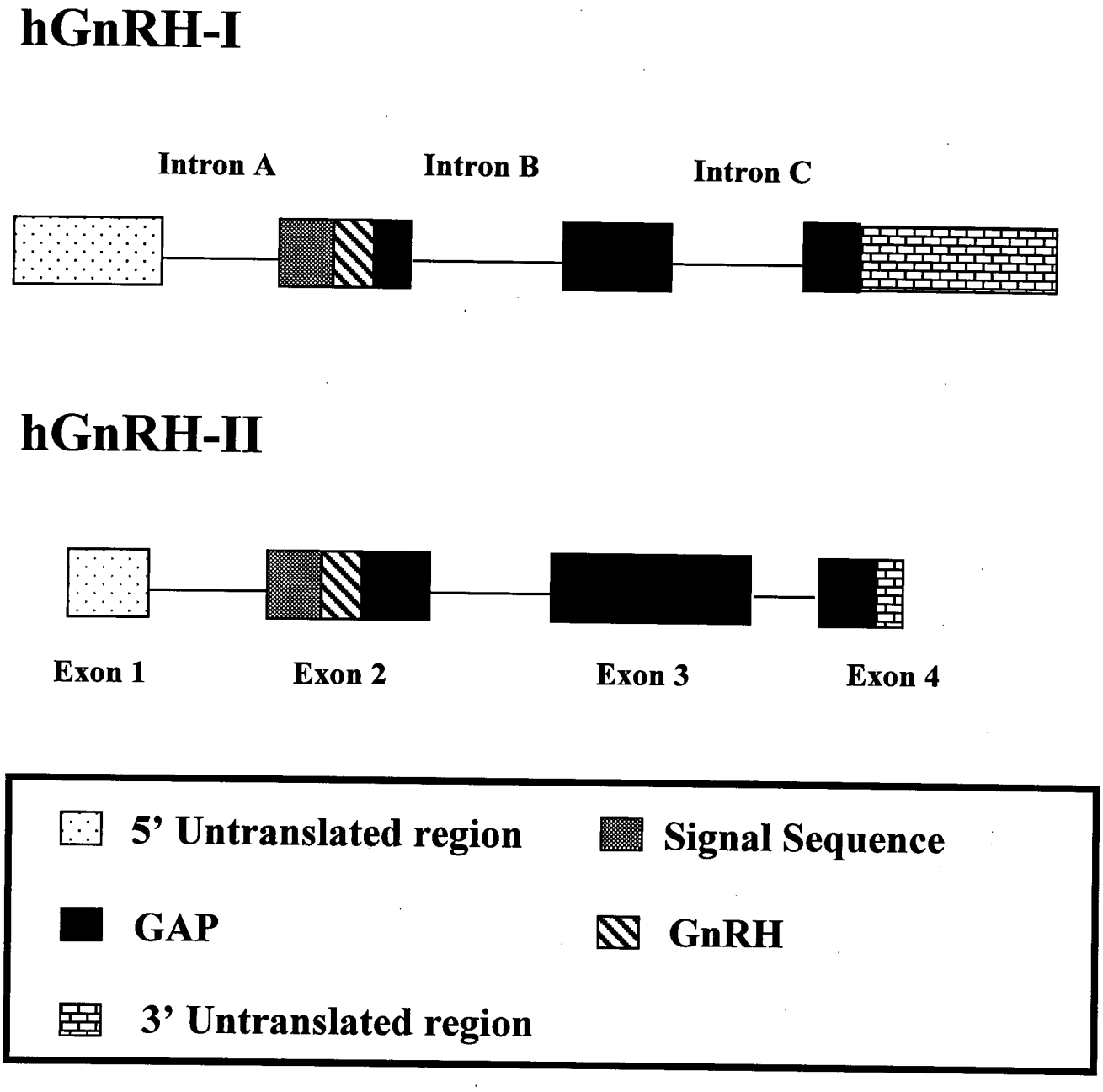


Figure 2. Schematic representation of the human GnRH-I and GnRH-II genes (not to scale). The structural similarity between the two genes reinforces their common ancestry. Both genes consist of four exons and three introns and encode for a preprohormone. The translated region encompasses exon 2-4 and the mature decapeptide is translated from the second exon. In humans, the gene for GnRH-I is located on chromosome 8, whereas the gene for GnRH-II is located on chromosome 20. Furthermore, the gene for GnRH-I (5.1 kb) is more then double the size of GnRH-II (2.1 kb). Adapted from White et al., 1998.

the mature peptide, GnRH (Jennes et al., 1997). Although the signal peptide is similar amongst the two forms of GnRH found in humans, the GAP is larger for GnRH-II when compared to GnRH-I (76 compared to 56 amino acids). Interestingly, White et al (1998) demonstrate that GnRH-II mRNA expression levels are signficantly higher outside the brain. Although GnRH-I mRNA has been detected in numerous extrapituitary tissues, including the human ovary, no information is available on GnRH-II expression in the ovary. Like GnRH-I, GnRH-II transcripts have variable lengths depending upon the tissue of origin. This indicates that the genes may contain multiple transcription initiation sites and undergoes differential post-transcriptional modifications, thereby providing evidence for tissue specific transcriptional regulation. Nevertheless, the amino acid sequence of the different forms of GnRH is highly conserved, reinforcing its important role in reproductive functions through evolution (Sherwood et al., 1993).

GnRH actions are mediated through a cell surface G-protein coupled receptor. The GnRH-R was initially cloned from the mouse pituitary gonadotrope cell line (αT3-1) (Tsutsumi et al., 1992; Ursula et al., 1997), and since then, has been cloned in sheep (Brooks et al., 1993), cow (Kakar et al., 1993), rats (Eidne et al., 1992; Kaiser et al., 1992; Kakar et al., 1994;) and humans (Chi et al., 1993, Kakar et al., 1992). In addition, the GnRH-R has been detected in many extrapituitary tissues including the brain (Marchetti et al., 1996), ovary (Kogo et al., 1995), leydig cells (Clayton et al., 1986; Bahk et al., 1995), placenta (Bramley et al., 1992), and specific tumors (Imai et al., 1994; Eidne et al., 1985; Gallagner et al., 1991; Bahk et al., 1998). In humans, the GnRH-R gene contains three exons and two introns (Figure 3) (Fan et al., 1994). Exon I encodes the 5' untranslated region and the first three transmembrane domains and a portion of the

fourth. Exon II encodes for the rest of transmembrane four and five and Exon III encodes for the rest of the open reading frame (Leung and Peng, 1996). The mature mRNA transcript translates into a 328 amino acid peptide in humans (Figure 4) (Fan et al., 1995) and is organized into an array of hydrophobic regions that correspond to the seven transmembrane domains, characteristic of all G-protein coupled receptors. However, unlike other G-protein coupled receptors, the GnRH-R lacks the intracellular carboxy terminal tail. Furthermore, the highly conserved Asp-Arg-Tyr (DRY) triplet found in the third transmembrane domain of many G-protein coupled receptors has been changed to Asp-Arg-Ser in the GnRH-R (Fan et al., 1995; Sealfon and Miller, 1995). In addition, the highly conserved aspartate and aspargines residues in the second and seventh transmembrane domain, respectively, of most G-protein coupled receptors have been exchanged in the GnRH-R (Stojikovic et al., 1994; Kaiser et al., 1997). The predicted molecular weight of the peptide is approximately 37.6 kDa. However, native GnRH-R migrates to 50-60kDa on electrophoretic profiles, indicating that the receptor undergoes post-translational modifications such as glycosylation and phosphorylation (Flanagan et al., 1997). Like its cognate ligand, the human pitutary GnRH-R displays strong homologies with other mammals including the mouse and rat, indicating its important role through evolution (Ursula et al., 1997).

Although the GnRH-R mRNA has been detected in extrapituitary tissues, including the human ovary, the molecular sequence of the full-length GnRH-R in the human ovary has not been characterized. Binding studies indicate that the GnRH-R in the gonads has a lower affinity then its pituitary counterpart (Kakar et al., 1994; Moumni

et al., 1994), suggesting that the primary receptor structure may be different in the ovary and the pituitary.

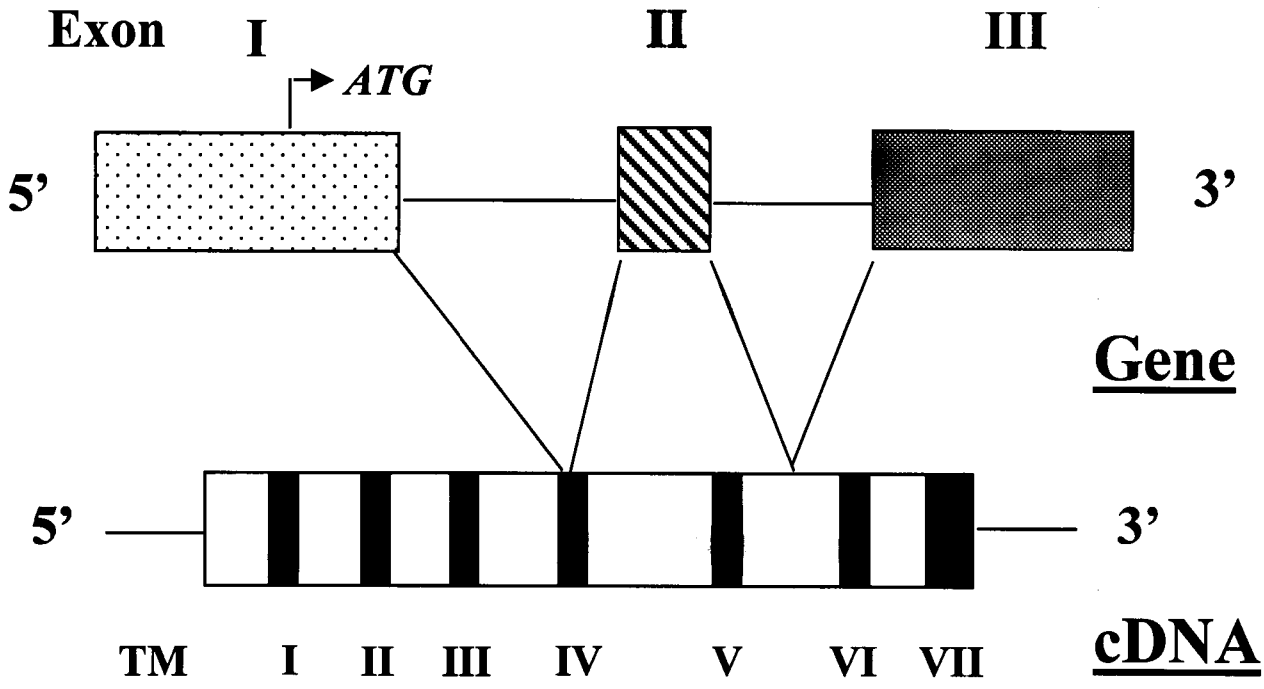


Figure 3. Schematic representation of the GnRH receptor gene and cDNA. The gene was first cloned in the mouse pituitary cells, and since then has been cloned in many other species. In humans the gene is located on chromosome 4 and encodes for a G-protein coupled receptor with the characteristic 7 transmembrane domains. The gene consists of three exons and two introns. The molecular structure of the GnRH-R is highly conserved throughout the animal kingdom, emphasizing the physiological importance of the GnRH-R.

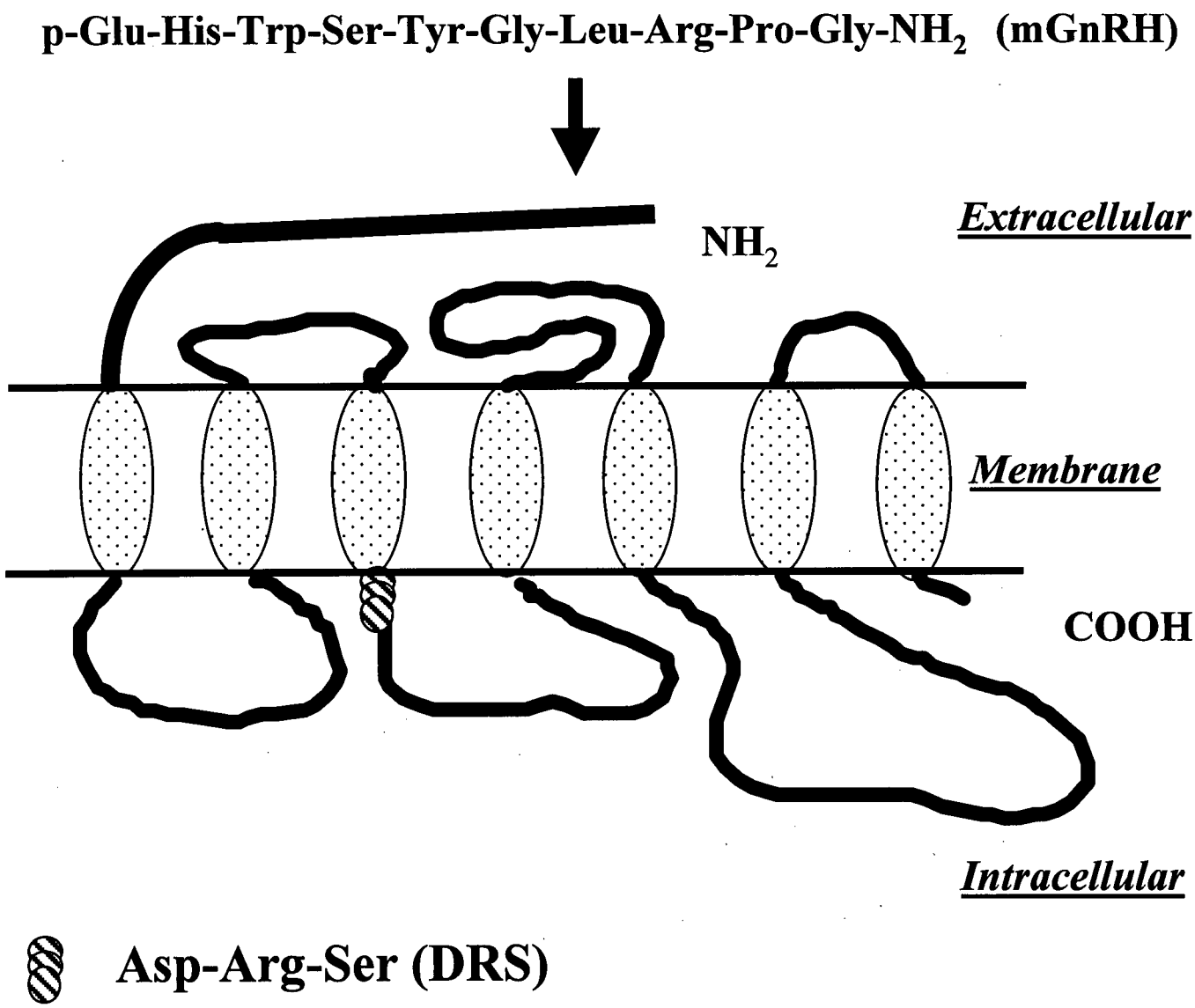


Figure 4. Primary structure of mammalian GnRH and its receptor (GnRH-R). In humans the mature mRNA transcript encodes for a 328 amino acid peptide. The primary sequence of the receptor is organized into an array of hydrophobic regions that correspond to the 7 transmembrane domains. Unlike other G-protein coupled receptor, the GnRH-R lacks a carboxy terminal tail. In addition, the highly conserved Asp-Arg-Tyr triplet found in the third transmembrane domain of most G-protein coupled receptors has been changed to Asp-Arg-Ser. Interestingly, conserved aspartate and asparagines residues in the second and seventh transmembrane domain have been exchanged. These unusual changes in the GnRH-R may have implications in signaling, receptor recycling and degradation.

VIII. GnRH Signalling

In the pituitary gland, binding of GnRH to its receptor leads to activation of two signal transduction pathways. Activation of protein kinase A through the adenylate cyclase effector system, or activation of protein kinase C through the phospholipase effector system (Leung and Steele, 1992; Kiesel, 1993). In contrast, the signal transduction pathway for GnRH in the ovary is complex and not well understood in the human. Activation of GnRH-R in the ovary leads to phospholipid metabolism. The involvement of the phospholipase C (PLC) pathway has been substantiated by the GnRH-induced release of intracellular calcium in rat and human granulosa cells (Leung et al., 1989; Hori et al., 1998). Activation of PLC is mediated through $G_{q/11\alpha}$, and leads to the production of inositol-1, 4, 5-triphosphate (IP_3) and diacylglycerol (DAG), culminating in the mobilization of intracellular calcium and activation of protein kinase C (PKC) respecitively. Other effector systems that are activated by GnRH in the ovary are phopholipase D (PLD) and phospholipase A_2 (PLA_2) (Leung and Steele, 1992). The PLD pathway is involved in the liberation of phosphotidic acid (PA) from phosphatidylcholine (PC). PA has been implicated in activation of PKC and mobilization of calcium. In contrast, PLA_2 generates arachidonic acid, a substrate for the lipoxygenase pathway (Leung and Steele, 1992).

There is evidence that the GnRH-R is coupled to $G_{i\alpha}$ in reproductive tract tumors (Iami et al., 1996; Limonta et al., 1999). In prostate cancer cells, GnRH led to an inhibition of cAMP accumulation (Limonta et al., 1999). In contrast, GnRH actions have been assoicated with activation of phosphotyrosine phosphatase in ovarian cancer cells,

which may be responsible for the GnRH-induced inhibition of growth (Imai et al., 1996). More recently, GnRH has been implicated in activating the MAP Kinase cascade in αT3-1 cells and rat pituitary cultures (Mitchell et al., 1995; Sim et al., 1995; Wolbers et al., 1995; Haisenleder et al., 1998). Our laboratory is currently investigating the role of GnRH-induced MAP kinase activation in extrapituitary tissues including the ovary, placenta and ovarian cancer cells.

IX. Regulation of GnRH and GnRH-R Gene Expression

Regulation of GnRH is under the control of factors in the hypothalamic-pituitary gonadal axis. The synthesis and secretion of GnRH is regulated through neuronal pathways that directly or indirectly regulate GnRH neurons. Factors such as cathecolamines, dopamine and other neuropeptides play a crucial role in regulating the frequency and amplitude of GnRH secretion (Sawyer, 1975). Autoregulation of GnRH has also been implicated in the rat hypothalamus. Through an ultrashort feedback loop, GnRH treatment inhibited secretion of GnRH into the rat hypothalamo-hypophyseal portal system (Valenca et al., 1987). *In vitro* studies using the GT1-7 neuronal cell line, demonstrate that GnRH regulates its own sythesis in a biphasic pattern (Krsamanovic et al., 1993). Gonadotropins from the pituitary gland influence GnRH in the hypothalamus in short feedback loops. However, hCG treatment had no significant effect on GnRH mRNA in hGLCs (Peng et al., 1994), indicating that the regulatory mechanisms are different in the ovary and the hypothalamus. Steroids also play a role in regulating GnRH gene expression (Ortmann et al., 1995; Rissman, 1996; Petersen et al., 1996). Estrogen treatment decreased levels of GnRH mRNA levels in the brain of ovarectomized rats. Accordingly, GnRH levels are inversely related to estrogen profiles during the rat estrus cycle. During early proestrus, E2 levels peak with concomitant low GnRH mRNA (Zoeller and Young, 1988). In contrast, estradiol treatment increased GnRH gene expression in the rat during the periovulatory period, resulting in the ovulatory gonadotropin surge (Park et al., 1990). The differences in GnRH regulation by gonadal steroids are undoubtedly explained by stage specificity, dose and duration of treatments.

However, no information is available on the role of steroids in regulating locally produced ovarian GnRH in the human.

Regulation of the GnRH-R has been well documented in the pituitary gland by various factors. The homologous regulation of GnRH-R by GnRH can have biphasic effects, depending on the time and dose of treatment (Mason et al., 1994; Kakar et al., 1994; Wu et al., 1994; Turzillo et al., 1995 Kakar et al., 1997). For example, in the mouse αT3-1 gonadotrope cell line, exposure to low concentrations of GnRH resulted in upregulation of receptor expression whereas, higher doses and chronic exposure lead to a significant decline in receptor expression. This decline was associated with receptor desensitization in the pituitary (Mason et al., 1994; Kakar et al., 1997; Ursula et al., 1997). Similarly, pulsatile GnRH administration increased GnRH-R number during luteolysis in the ewe pituitary (Turzillo et al., 1995). Furthermore, the GnRH-R levels varied during the estrus cycle where the number of receptors was greatest just before the onset of the gonadotropin surge, and was sustained throughout the surge (Kakar et al., 1994). In contrast, there was a significant decline in GnRH-R expression during lactation and pregnancy in the rat pituitary (Funabashi et al., 1994).

Steroid hormones also affect GnRH-R expression in the pituitary of many species (Gregg et al., 1990; Laws et al., 1990; Emons G et al., 1992; McArdle et al., 1992; Yasin et al., 1995; Adams et al., 1996; Quinones-Jenab et al., 1996; Sakurai et al., 1997). In ovine, estrogen increased and progesterone decreased GnRH-R number (Gregg et al., 1990; Laws et al., 1990). Analogously, administration of estradiol to ovarectomized rats, increased GnRH-R expression in the pituitary (Quinones-Jenab et al., 1996). However, other groups have shown that ovariectomy increased GnRH-R mRNA levels, followed by

a significant decrease with estrogen replacement therapy in rat pituitaries (Kaiser et al., 1993). Other factors such as inhibins, activins and prostoglandins may also play a crucial role in GnRH-R regulation (Braden et al., 1990; Laws et al., 1990; Braden and Conn, 1992; Turzillo et al., 1997). For example, inhibin increased GnRHR number in the ovine pituitary and decreased GnRH-R number in the rat pituitary (Laws et al., 1990; Gregg et al., 1991). Differences in GnRH-R regulation may be explained by species and stage specific regulation.

Unlike the pituitary, regulation of GnRH and its receptor is not well understood in the ovary. In the rat ovary, in situ hybridization revealed that GnRH-R expression was dependent on the stage of the estrus cycle (Bauer-Danton and Jameson, 1995). The receptor expression was greatest in preovulatory follicles and atretic follicles, followed by the corpus luteum and smaller follicles (Whitelaw et al., 1995). Interestingly, GnRH-R expression in the rat ovary correlated with receptor expression in the pituitary. The highest level of receptor expression was in proestrus, just prior to the gonadotropin surge and was maintained throughout the gonadotropin surge, followed by a decline in metestrus (Bauer-Danton and Jameson, 1995). In preovulatory rat granulosa cells, GnRH induced a dose-dependent increase in its receptor whereas LH induced a decrease in GnRH-R transcripts (Olofsson et al., 1995). However, very little information is available for the primate ovary. Recently, GnRH and its receptor have been detected in human granulosa luteal cells (Peng et al., 1994). GnRH induced a biphasic effect on GnRH and GnRH-R gene expression in these cells. At a 1 nM dose of GnRH, there was a significant increase in GnRH and GnRH-R gene expression whereas, at higher doses there seemed to be an inhibitory effect. Correspondingly, progesterone levels declined at

1nM treatment of GnRH, providing supporting evidence that GnRH acts as an autocrine/paracrine modulator in the ovary. Furthermore, gonadotropin treatment (hCG) in hGLCs inhibited GnRH-R gene expression (Peng et al., 1994). However, no information is available on the role of gonadal steroids in regulating GnRH-R gene expression in the human ovary.

X. Clinical Applications of GnRH

Clinically, GnRH and its analogues have been used to treat numerous reproductive disorders. In Assisted Reproductive Technologies (ART), suppression of endogenous gonadotropin secretion by GnRH agonists (GnRHa), followed by exogenous administration of gonadotropins is a common practice in ovulation induction protocols. In addition, GnRHa is used to treat precious puberty, endometriosis and specific reproductive tract cancers such as prostate cancer. However, the aformentioned treament regimens are based on the down-regulation effects at the level of the hypothalamus and pituitary gland. With the recent detection of GnRH and its receptor in extrapituitary tissues including the ovary, direct ovarian effects of GnRH agonists on follicle development and quality have been raised (Lefevre et al., 1991; Testart et al., 1993). Thus, understanding the dynamics of the intrinsic ovarian GnRH axis may play an important role in the discernment of ART outcomes.

B. Rationale

Regulation of GnRH-I and its Receptor mRNA

Through a receptor mediated process, hypothalamic GnRH stimulates the pituitary gonadotropes to synthesize and secrete gonadotropins. Recently, GnRH and its receptor have been detected in numerous extrapituitary tissues, including the primate ovary (Clayton et al., 1979; Peng et al., 1994; Minaretzis et al., 1995). However, the physiological role of GnRH in the ovary is not well understood. Although regulation of hypothalamic GnRH and its receptor have been well characterized, the regulation of the intrinsic ovarian GnRH axis remains poorly understood. As gonadal steroids are key regulators of reproductive functions, the present study examined the effect of steroids on GnRH and GnRH-R in hGLCs in an attempt to understand the physiological role that GnRH plays in the human ovary.

Expression and Function of a second form of GnRH in the human ovary

Multiple forms of GnRH have been identified throughout the animal kingdom. Within the same species, more then one form of GnRH may exist and the variable forms may have distinct functions (Montero et al., 1995; Schalburg et al., 1999). Despite previous reports indicating that placental mammals possess at atleast two forms of GnRH, until recently, it was thought that reproduction in primates was controlled by only one form of GnRH, namely mammalian GnRH (mGnRH). A second form of GnRH, analagous to chicken II GnRH (cGnRH-II), has been isolated from the primate brain (Lescheid et al., 1997). However, tissue distribution studies indicated that GnRH-II expression levels were significantly higher outside the brain, specifically in the prostate and kidney (White et al., 1998). In view of the expression of GnRH-II in the human

brain and expression of multiple forms of GnRH in the ovary of other species, we examined the expression and function of a second form of GnRH in the human ovary.

C. Hypothesis

Gonadotropin-Releasing Hormone and its receptor mRNA will be dynamically regulated during spontaneous luteinization and by locally produced gonadal steroids during the luteal phase of the menstrual cycle. Furthermore, the expression and function of GnRH-II in the human ovary will further strengthen the notion that GnRH is an autocrine/paracrine regulator of ovarian function in the human.

D. Specific Objectives

1. To characterize the molecular structure of ovarian GnRH-R cDNA and compare it to its pituitary counterpart.
2. To examine the changes in GnRH and GnRH-R gene expression during spontaneous luteinization *in vitro*
3. To determine if GnRH and GnRH-R mRNA are regulated by gonadal steroids (estradiol and progesterone) in hGLCs (time course and dose response studies).
4. To examine the expression and function of a second form of GnRH (GnRH-II) in the human ovary.

E. MATERIALS AND METHODS

I. Granulosa-Luteal Cell Culture and Pharmacological Treatments

The use of human granulosa-luteal cells *in vitro* was approved by the Clinical Screening Committee for Research and Other Studies involving Human Subjects of the University of British Columbia. Follicular aspirates were collected during oocyte retrieval from women undergoing *in vitro* fertilization at the University of British Columbia and granulosa-luteal cells were prepared as previously described (Peng et al., 1994), with some modifications. Briefly, the follicular contents were centrifuged at 1000 xg and the supernatant was removed. The cells were resuspended in 6 ml of medium 199 (M199) (Gibco, Burlington, Ontario, Canada), layered onto Ficoll Paque (Pharmacia Biotech, Morgan, Canada) and centrifuged to remove red blood cells. Cells in the interface were collected and rinsed twice with M199 supplemented with 100 U/ml penicillin G and 100 μg/ml streptomycin. The cell pellet was resuspended in M199 supplemented with 10 % fetal bovine serum (FBS), 100 U/ml penicillin G and 100 μg/ml streptomycin at a density of $1x10^5$ cells/ml. The cells were seeded at a density of $2x10^5$ cells in 35 mm culture dishes, and were allowed to adhere for 48 h at 37 C in a humidified atmosphere of 5 % CO_2-95 % air. Subsequently, the cells were rinsed with Hanks Balanced Salt Solution (HBSS), and on day 4 in culture the cells were transferred into phenol red-free M199 supplemented with 2 % charcoal-stripped FBS. Cells were treated with estradiol or RU486 in a time- and dose-dependent manner in phenol red-free M199 on day 5 in culture. Control cultures were treated with vehicle (0.01% w/v of ethanol). In addition, tamoxifen treatment alone was also performed in the appropriate studies.

II. Total RNA Extraction and First Strand cDNA synthesis

Total RNA was extracted from cultured hGLCs using the RNaid Kit (Bio/Can Scientific, Mississauga, Canada) according to the manufacturer's protocol. Briefly, cells were disrupted in lysis buffer [4 M guanidine thiocynate, 25 mM sodium citrate (pH 7.0), 0.5% N-lauroyl sarcosine, and 0.1 M β-mercaptoethanol] and then acid phenol extracted. RNA was purified from the aqueous phase on an RNA matrix and eluted into ribonuclease-free water. The concentration of RNA was determined by the absorbance at 260 nm and the integrity was confirmed by agarose-formaldehyde gel electrophoresis.

III. Cloning and Sequencing of the Full Length GnRH-R cDNA

One microgram of total RNA was reverse transcribed into first strand cDNA in a total volume of 15 μl using the first strand cDNA synthesis kit (Amersham Pharmacia Biotech, Laval, Quebec, Canada). Based on the published pituitary sequence for human GnRH-R, two primers were designed to amplify the full length GnRH-R from hGLCs. The sense and antisense primers were 5'-AATATGGCAAACAGTGCCTCTC-3' (P48-2) and 5'-CAATCACAGAGAAAAATATCCA-3' (P48R), respectively. The cDNA was amplified in a 50 μl PCR reaction containing 2.5 units of Taq polymerase (Gibco-BRL Life Technologies, Burlington, Canada) and its buffer, 1.5 mM $MgCl_2$, 10 mM each dNTP and 50 pmol of each primer. The PCR product was separated by agarose gel electrophoresis and visualized with ethidium bromide staining and ultraviolet light. The PCR product was transferred to a nylon membrane and probed with a digoxigenein-labelled GnRH-R cDNA probe (Boehringer Mannheim, Germany). All probes used for Southern blot analysis were internal to the oligonucleotide primers used for PCR

amplification to avoid non-specific binding. After high stringency washes, the membrane was exposed to Kodak Omat X-ray film. The putative full-length GnRH-R cDNA was cloned into PCRII Vector (InVitrogen, USA) and sequenced by the dideoxy nucleotide chain termination method using the T7 DNA Polymerase Sequencing Kit (Amersham Pharmacia Biotech).

IV. RT-PCR and Quantification of GnRH mRNA from hGLCs

Using a semi-quantitative PCR system, GnRH mRNA levels were quantitated. PCR amplification was carried out in 50 μl reactions containing 2.5 units of Taq polymerase (Gibco-BRL Life Technologies, Burlington, Canada) and its buffer, 1.5 mM $MgCl_2$, 2mM each dNTP and 50 pmol of sense and antisense primer. The primers for GnRH were designed based on the published sequence for human hypothalamic GnRH. The forward and reverse primers were 5'-ATTCTACTGACTTGGTGCGTG-3' (F1) and 5'-GGAATATGTGCAACTTGGTGT-3' (R1), respectively. PCR amplification was carried out for 26 cycles with denaturing at 94 C for 60 sec, annealing at 53 C for 35 sec and extension at 72 C for 90 sec, followed by a final extension at 72 C for 15 min. To standardize for first strand cDNA synthesis efficiency, PCR for β-actin was performed. Primers for β-actin were designed according to the published sequence (Ng et al., 1985). Amplified PCR products were separated by agarose gel electrophoresis and subjected to Southern blot analysis. Quantitation was performed using a visual light densitometer (Model 620, Bio-Rad Laboratories, Richmond, CA).

V. Construction of the native (target) and mutant (competitive) cDNA for GnRH-R

Using an internal primer pair (P44F and P45R) based on the human pituitary GnRH-R cDNA, a 347 bp fragment of native GnRH-R was obtained by PCR amplification from human pituitary cDNA. The primers were sense 5'-GTATGCTGGAGAGTTACTCTGCA-3' (P44F) and antisense 5'-GGATGATGAAGAGGCAGCTGAAG-3' (P45R). The expected 347 bp PCR product was separated by agarose gel electrophoresis and confirmed to be hGnRH-R by Southern blot analysis. The PCR product was cloned into PCRII cloning vector (Invitrogen, USA). Sequence analysis was performed to confirm the identity of the cloned fragment. Subsequently, the fragment was subcloned into pBSKII (Stratagene, USA) at Sac I and Xho I sites. The mutant competitor cDNA was generated by digesting the subcloned fragment with Hind III and Sty I and self-ligating the resulting clone. The 227 bp hGnRH-R cDNA fragment contained a 120 bp deletion from the native clone. However, the competitor retained the identical primer binding sites as the native GnRH-R cDNA.

VI. RT-PCR and Quantification of GnRH-R mRNA from hGLCs

For competitive PCR, 4 μl of first strand cDNA from 1 μg of total RNA was co-amplified with 0.08 pg of mutant GnRH-R cDNA. PCR amplification was carried out in 50 μl reactions containing 2.5 units of Taq polymerase (Gibco-BRL Life Technologies, Burlington, Canada) and its buffer, 1.5 mM $MgCl_2$, 2 mM each dNTP and 50 pmol of sense (P44F) and antisense primer (P45R). Amplified PCR products were separated by agarose gel electrophoresis and subjected to Southern blot analysis. The PCR products were hybridized with a digoxigenin-labelled GnRH-R cDNA probe (Boehringer

Mannheim, Lavel, Canada). After stringency washes, the membranes were exposed to Kodak Omat X-ray film. Quantitation was performed using a visual light densitometer (Model 620, Bio-Rad Laboratories, Richmond, CA).

VII. Expression of GnRH-II in hGLCs

Human GCs were cultured and maintained as aformentioned. Primary ovarian cancer cells and ovarian surface epithelium (OSE) were isolated and maintained as previously described (Kang et al., 2000). Ovarian cancer cell line, OVCAR-3, was cultured at 37 C in M199 suplemented with 5% fetal bovine serum (FBS), 100 U/ml penicillin G and 100 μg/ml streptomycin (Gibco-BRL Life Technologies). Total RNA was extracted from the ovarian cells using the RNaid Kit (Bio/Can Scientific, Mississauga, Canada) as per the manufacturer's protocol. Based on the published sequences for human GnRH-II, primers were designed to amplify GnRH-II from the ovarian cells. The sense and antisense primers for cGnRH-II were 5'-GCCCACCTTGGACCCTCAGAG-3' (GIIF) and 5'-CCAGGTGTCGCTTCCTGTGAA-3' (GIIR), respectively. The cDNA was synthesized from 1ug of total RNA (Pharmacia, Biotech, Morgan, Canada), and amplified in a 50μl PCR reaction containing 2.5units of Taq polymerase (Gibco-BRL Life Technologies, Burlington, Canada) and its buffer, 1.5mM $MgCl_2$, 10mM each dNTP and 50 pmole of each primer. PCR amplification was carried out for 35 cycles with a denaturation step at 94 C for 60s, followed by annealing at 60 C and extension for 90s at 72 C. The PCR products were subjected to agarose gel electrophoresis and ethidium bromide staining. The expected size fragments were gel purified and cloned into PCRII Vector (InVitrogen,

USA) and sequenced by the dideoxy nucleotide chain termination method using the T7 DNA Polymerase Sequencing Kit (Pharmacia Biotech, Morgan, Canada).

VIII. Progesterone Radioimmunoassays (RIAs)

Human GCs were seeded at a density of 1.5 x 10^5 cells in 35mm dishes. On day 5 in culture, hGLCs were treated with 10^{-7} M GnRH-I or GnRH-II alone or in combination with antide (10μM) in duplicates for 24 h. The media was removed and stored at –20°C and subsequently assayed for progesterone content. The cells were lysed with 80 ul of RIPA (containing 1X PBS (pH 7.4), 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 10 μg/ml PMSF, 30 μg/ml aprotinin and 10 μg/ml leupeptin), and the cellular lysates were incubated on ice for 10 min prior to centrifugation. The supernatant was collected and assayed for total protein content using a modified Bradford Assay (Bio-Rad) to standardize for cell number. The RIA for progesterone was performed as previously described (Goff et al., 1979; Li et al., 1993). Briefly, the samples were incubated with 0.05 mg rabbit P4 antisera (kindly provided by Dr. DT Armstrong, University of Western Ontario) and 20,000 cpm of tracer ([1,2,6,7,6,17-^{3}H] progesterone; Amersham) per tube. The range of standards was from 0.38-100 ng/ml. All assay contents were diluted with 0.1 M phosphate buffered saline (PBS-G) (containing 8.8 g/L Na_2HPO_4, 5.3 g/L NaH_2PO_4:H_2O, 0.1 % gelatin) to a final assay volume of 300 μl. After 16-24h incubation at 4°C, 500 μl of a cold charcoal:dextran solution (0.25:0.025 %w/v in PBS-G) was added to each tube and vortexed to separate bound from unbound antigen. After centifugation, 500 μl of the supernatant was removed and diluted with 3 ml of scintillation cocktail (BDH) and counted in a Wallac 1217 Rack beta-counter. The

standard curve and samples were assayed in triplicate. Inter- and Intra-assay coefficients of variation were less then 10%.

IX. Data Analysis

Relative GnRH mRNA levels were represented by the ratio of GnRH to β-actin. The amount of GnRH-R transcript was calculated based on the ratio of the target to competitive cDNA. The data are represented as the percent change relative to control. Data are depicted as the mean +/- standard error (SE). Each experiment was conducted with four different patient samples. For the RIA's, data are represented as a percent change relative to controls, and were standardized against total protein content. Each experiment was conducted in duplicates with four different patient samples. The data were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey's multiple comparison test (PRISM Graphpad Version 2, Graphpad Software, Inc., San Diego, USA). A $P<0.05$ was considered statistically significant.

F. RESULTS

I. Expression and Cloning of the ovarian GnRH-R cDNA from hGLCs

RT-PCR amplification was used to investigate the expression of the ovarian GnRH-R transcript equivalent to the pituitary form from hGLCs. A pair of primers encompassing the start and stop codons was designed based on the human pituitary sequence of GnRH-R cDNA (Fig. 5A). As shown in Fig 5B, PCR amplification yielded a 1003 bp fragment. Upon transfer of the PCR product to a nylon membrane and hybridization with a specific cDNA probe, a positive signal was detected, thereby confirming the identity of the PCR product (Fig. 5B, lower panel). The possibility of cross-contamination was ruled out, as no PCR product was detected in the negative control (lane 1, Fig 5B). Since the primers were located in different exons, the amplified product was not due to genomic DNA contamination, but rather specific amplification of mRNA. Sequencing analysis revealed that the GnRH-R from hGLCs was identical the published human pituitary cDNA sequence (data not shown).

II. Validation of semi-quantitative and competitive RT-PCR for GnRH and GnRH-R

Using semi-quantitative RT-PCR with primers derived from the human hypothalamic GnRH cDNA (Fig .6A), the levels of GnRH mRNA were examined in hGLCs. To determine where PCR amplification for GnRH and β-actin mRNA were in the logarithmic phase, 1 μg of total RNA was reverse transcribed and amplified under different cycle numbers (Figs. 6B, 7). Southern blot analysis revealed a 380 bp PCR product corresponding to GnRH, and a 525 bp PCR product for β-actin. A linear

relationship was observed for both GnRH (Fig. 6B, lower panel) and β-actin (Fig. 7) mRNA amplification with varying cycle numbers. Consequently, 26 cycles for GnRH and 18 cycles for β-actin were employed for quantification purposes. GnRH-R mRNA levels were analyzed using competitive RT-PCR (Fig. 8A). Co-amplification of the native and mutant GnRH-R cDNA yielded two PCR products at 347 bp and 227 bp, respectively (Fig. 8B). A standard curve for GnRH-R was constructed by co-amplification of a fixed amount of mutant GnRH-R cDNA with serial dilutions of the native GnRH-R construct. Increasing the amount of native construct resulted in a decreased amplification of the mutant internal standard (Fig. 9A). When the native GnRH-R cDNA was plotted as a function of the ratio of the native and mutant GnRH-R construct, a linear relationship was observed (Fig. 9B). To determine the optimal concentration of the competitor for quantification purposes, a fixed amount of first strand cDNA (4ul synthesized from 1 μg of total RNA) was co-amplified with serial dilutions of the competitor (data not shown). Increasing the amount of mutant construct resulted in a decline of native GnRH-R amplification from the cDNA sample. For quantitation purposes, 0.08 pg of mutant construct was employed for competitive RT-PCR for GnRH-R.

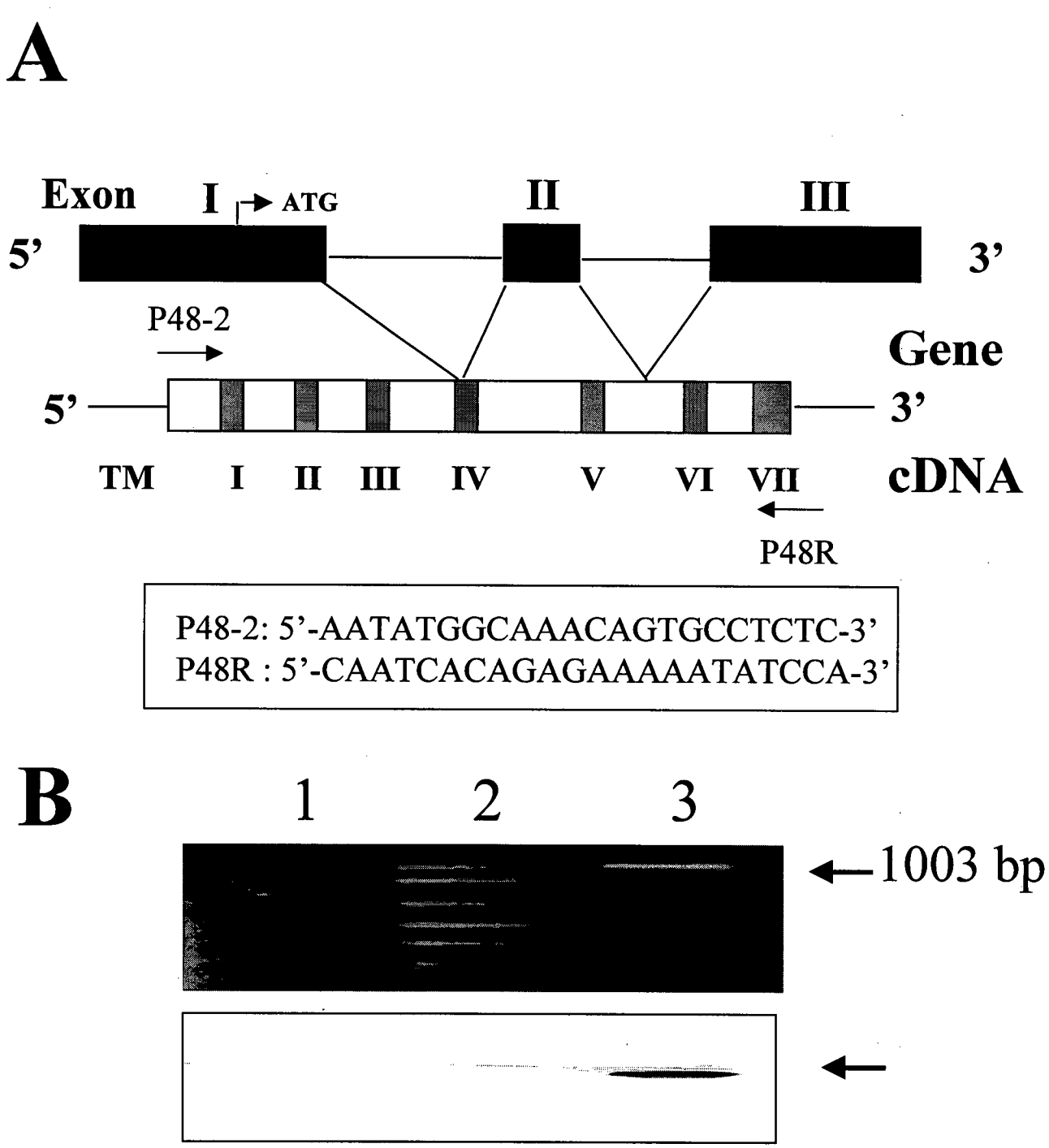


Figure 5. Molecular characterization of the full-length GnRH-R cDNA from hGLCs. (A). Schematic representation of the GnRH-R gene and cDNA. The positions and sequences of the primers used for RT-PCR amplification of the coding region of GnRH-R are depicted. TM, transmembrane domain. (B). RT-PCR amplification and Southern blot analysis of the GnRH-R from hGLCs. One microgram of total RNA was reverse transcribed and amplified for 35 cycles. A negative control without cDNA template was used to control for PCR contamination (Lane 1). The PCR product was resolved on a 1% agarose gel stained with ethidium bromide and a 1003-bp fragment was visualized (Lane 3) (upper panel). MW, molecular weight marker (Lane 2). The 1 kb fragment was confirmed to be GnRH-R by southern blot analysis (lower panel).

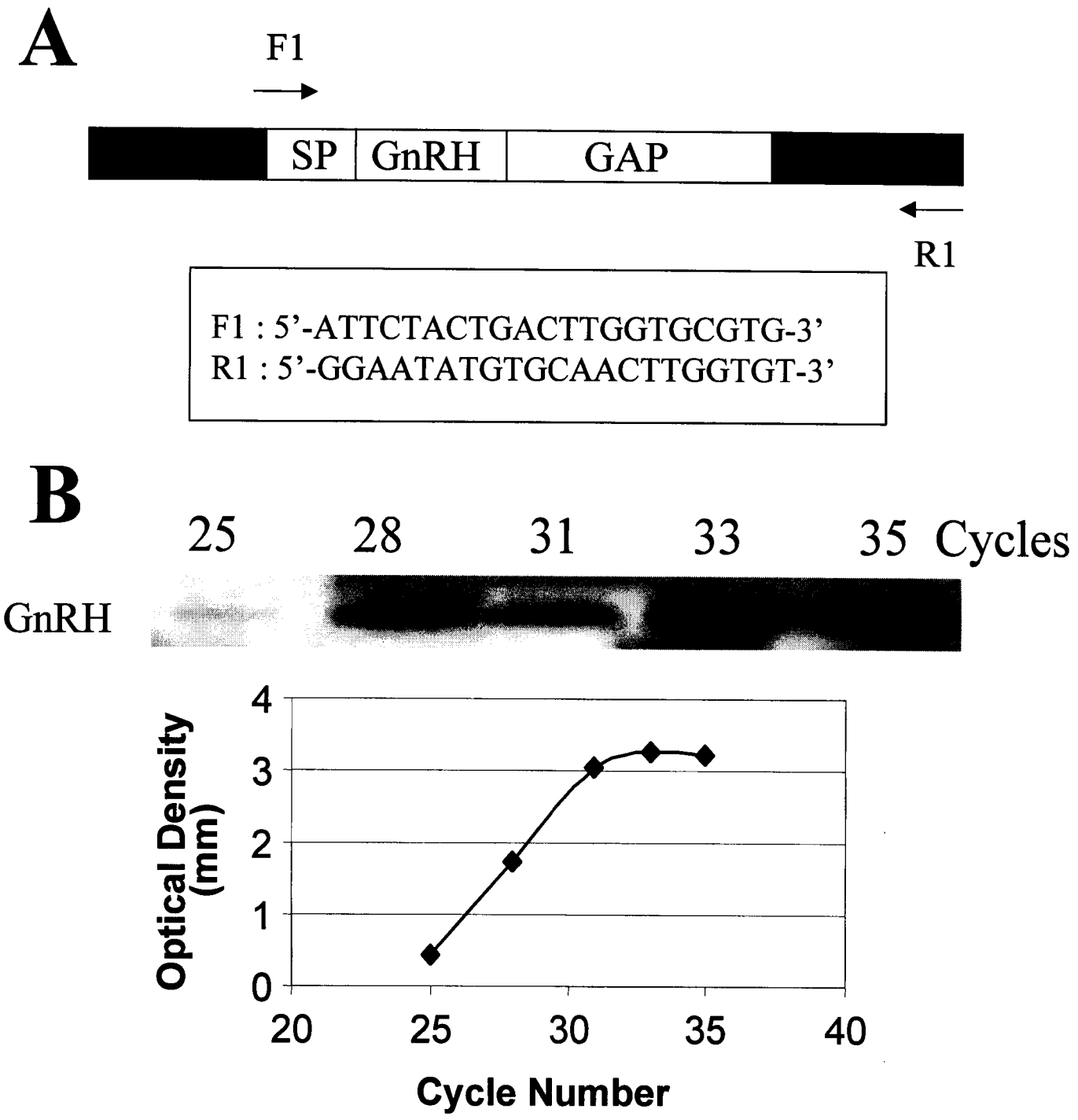


Figure 6. Validation of semi-quantitative RT-PCR for GnRH from hGLCs. (A). Schematic representation of the GnRH cDNA, including primer positions and sequences. SP, signal peptide; GAP, GnRH associated peptide. (B). Total RNA was extracted from hGLCs and 1 μg was reverse transcribed. Subsequent to PCR amplification with primers F1/R1, a 380-bp fragment was detected by agarose gel electrophoresis. To determine the linear phase of PCR amplification, GnRH was amplified from hGLCs cDNA under increasing cycle numbers. A linear relationship between the cycle number and optical density was observed between 25-30 cycles. Hence, 26 cycles was used for quantitation of GnRH mRNA from hGLCs

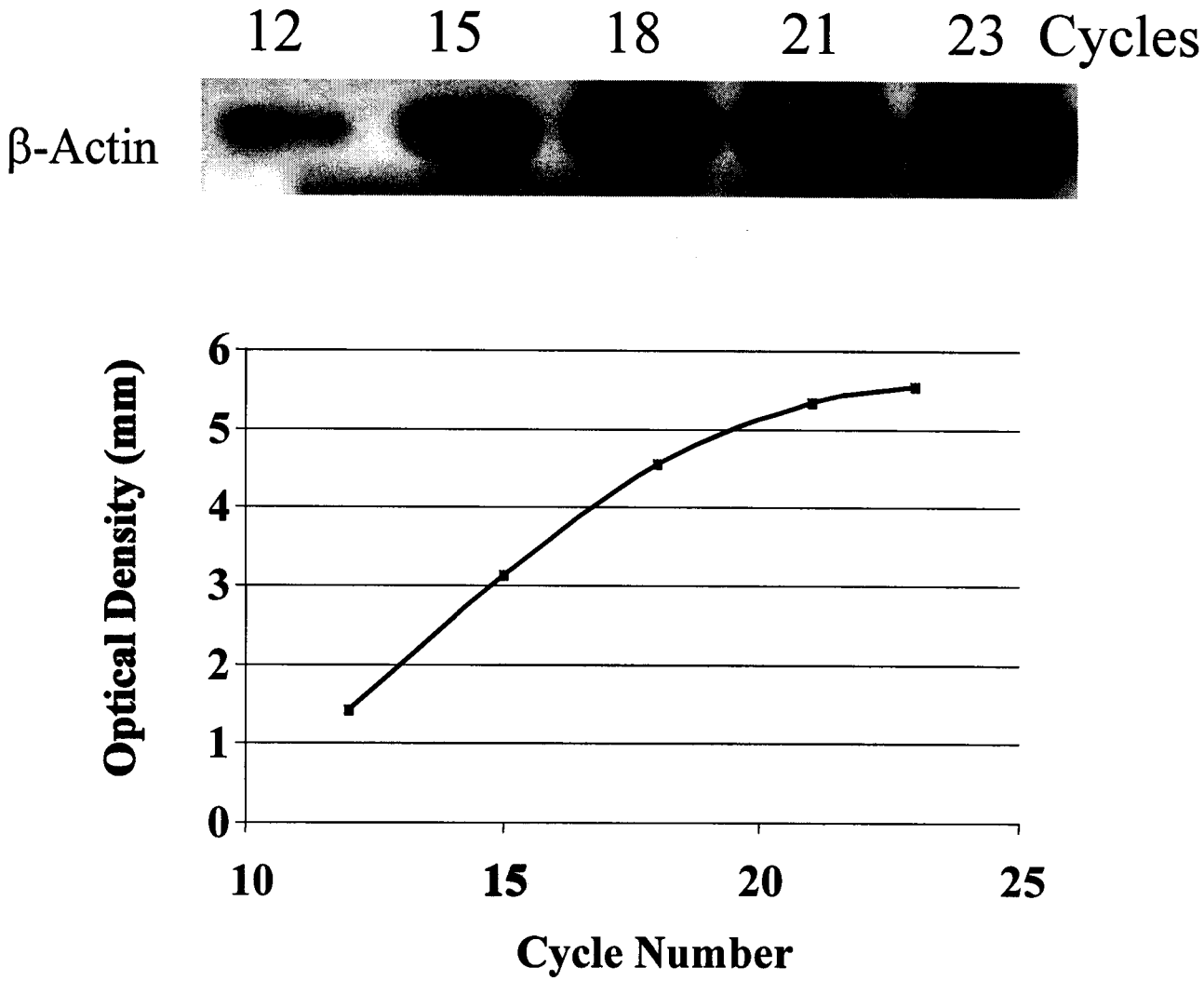


Figure 7. Validation of semi-quantitative RT-PCR for β-actin from hGLCs. Total RNA was extracted from hGLCs and 1 μg was reverse transcribed. Subsequent to PCR amplification with primers designed from the published sequence, a 525-bp fragment was detected by agarose gel electrophoresis. To determine the linear phase of PCR amplification, β-actin was amplified from hGLCs cDNA under increasing cycle numbers. A linear relationship between the cycle number and optical density was observed between 15-20 cycles. Hence, 18 cycles was used for quantitation of β-actin mRNA from hGLCs

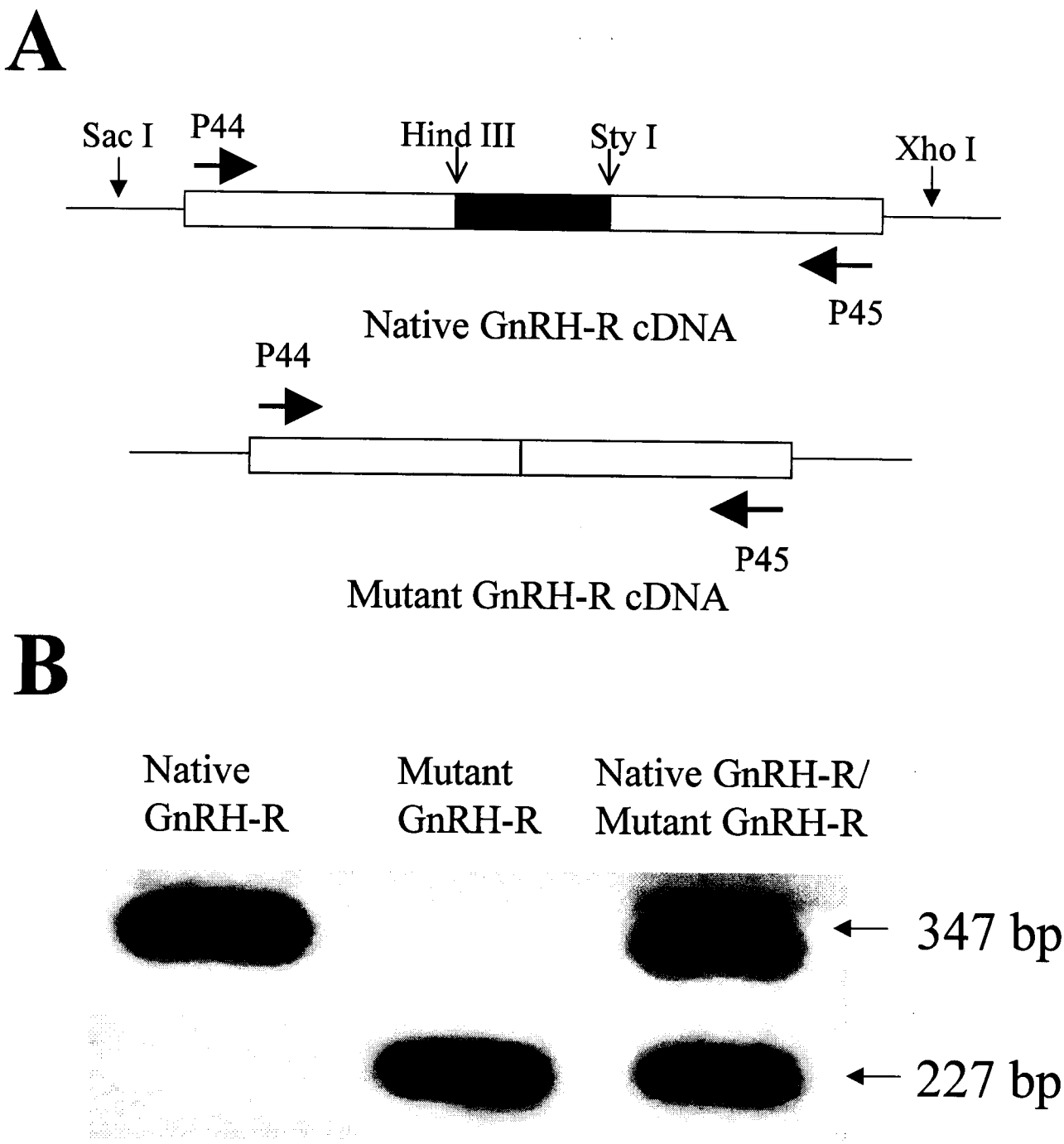
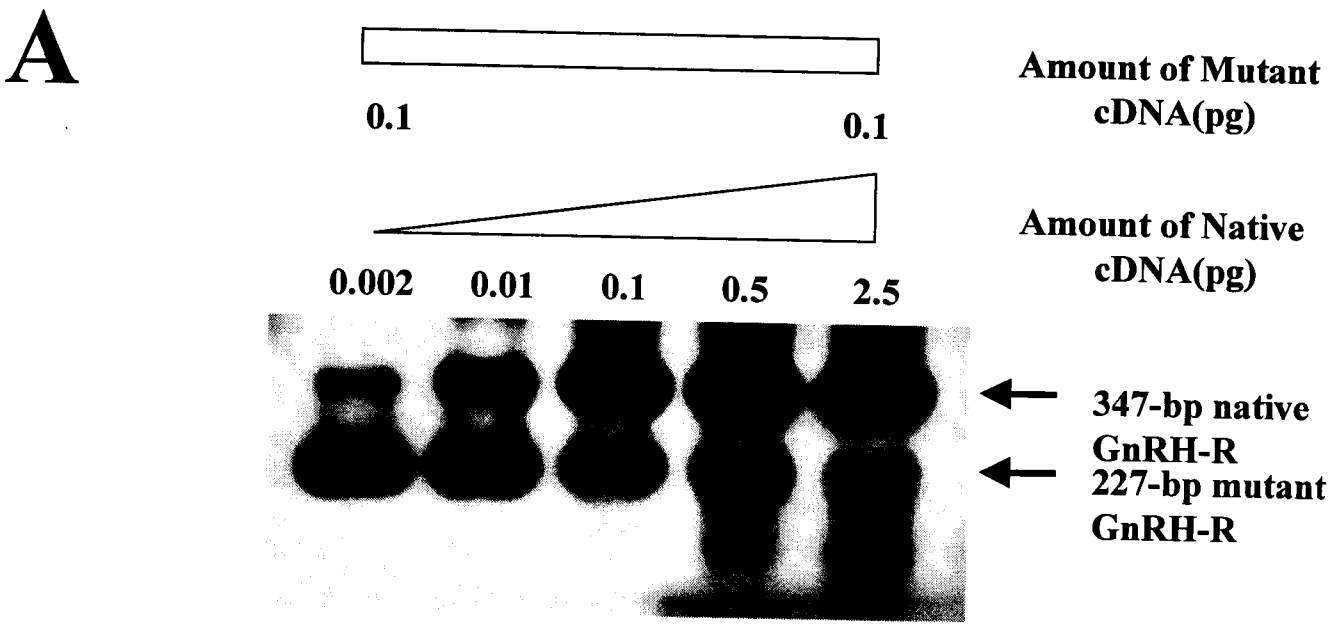


Figure 8. Construction of the native and mutant GnRH-R cDNA for competitive RT-PCR. (A). Internal primers (P44F and P45R) were designed based on the published human GnRH-R cDNA sequence from the pituitary gland. RT-PCR amplification from a 760-bp human pituitary cDNA template resulted in a 347-bp fragment corresponding to the native GnRH-R cDNA construct. The fragment was subcloned into pBSK II (Stratagene, USA) at Sac I and Xho I restriction enzyme sites. The mutant was generated by digesting the native GnRH-R cDNA with Hind III and Sty I, followed by self ligation. (B). PCR amplification of the native and mutant GnRH-R cDNA with P44 and P45 resulted in the expected 347-bp and 227-bp fragments, respectively (Lanes 1 and 2). Also note that both the native and mutant GnRH-R were co-amplified in the same PCR reaction (Lane 3).



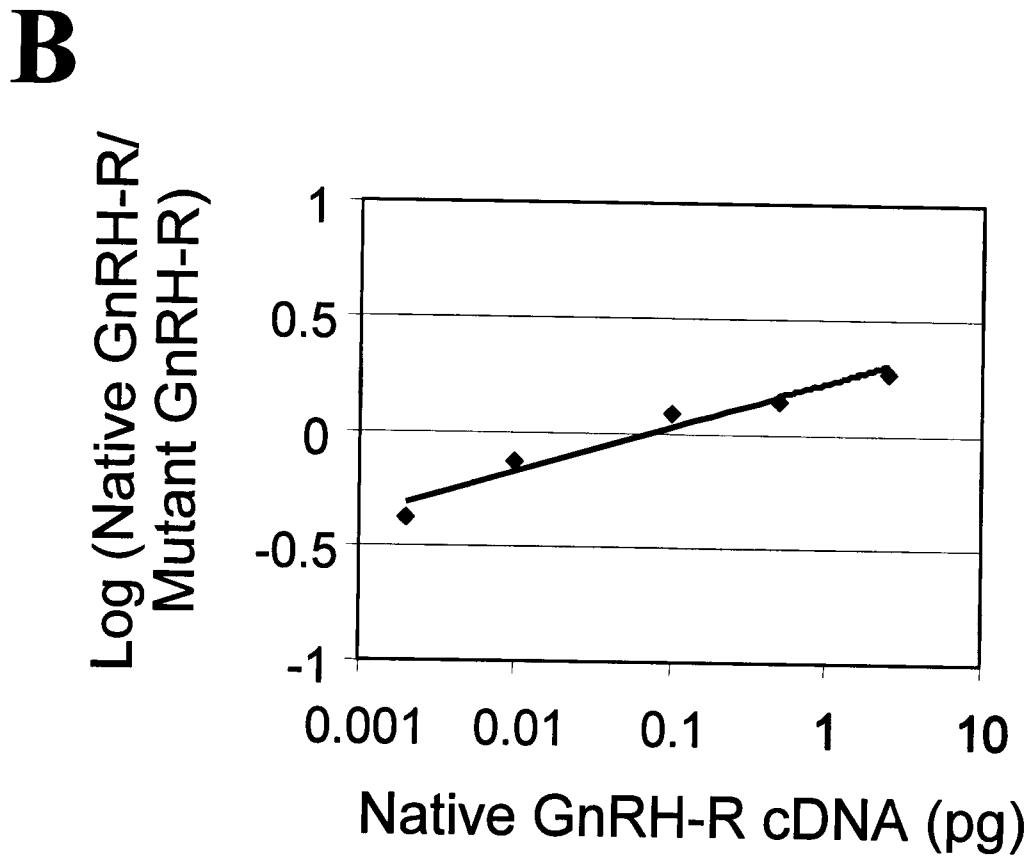


Figure 9. Validation of competitive RT-PCR for GnRH-R. (A). The standard curve for GnRH-R competitive PCR was constructed by co-amplification of a fixed amount of mutant GnRH-R cDNA and varying concentrations of the native GnRH-R cDNA. PCR amplification was carried out for 33 cycles, and the products were analyzed by Southern blot analysis. (B). A linear relationship was observed when the ratio of the native and mutant GnRH-R was plotted against the concentration of native cDNA.

III. Expression of GnRH and GnRH-R during spontaneous luteinization *in vitro*

To examine the changes in GnRH and GnRH-R mRNA levels with time in culture, hGLCs were cultured for 1, 4, 8 and 10 days followed by RNA extraction and RT-PCR quantitative analysis. As seen in Figs. 10 and 11, GnRH and its receptor mRNA levels increased significantly ($p<0.05$) with time in culture. For quantitative purposes, all time points were standardized to day 1 mRNA levels. Hence the day 1 cultures acted as controls. For GnRH mRNA levels (Fig. 10), a 65% increase ($p<0.05$) was observed on day 10 compared to day 1 cultures. When day 4 cultures were compared to day 8 and 10, there was a significant increase in GnRH mRNA levels with increasing time in culture. However, there was no significant difference between day 8 and day 10 cultures for GnRH mRNA levels. A similar trend was observed for GnRH-R mRNA levels (Fig. 11). There was a 55% and 63% ($p<0.05$) increase in GnRH-R mRNA levels on day 8 and 10 compared to day 1 cultures, respectively.

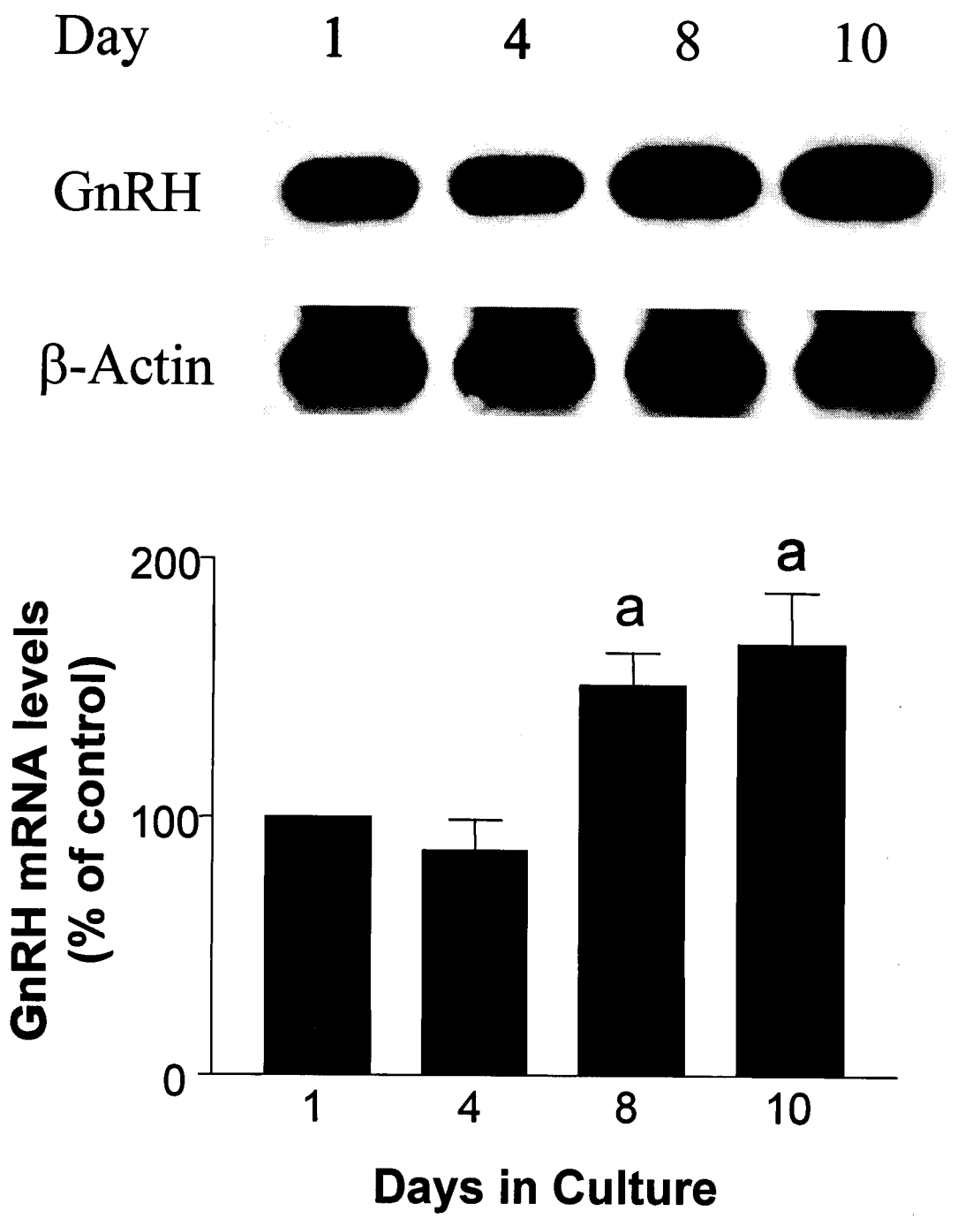


Figure 10. Changes in GnRH mRNA expression with spontaneous luteinization in culture. Total RNA was extracted from hGLCs (n=4) on day 1, 4, 8 and 10 in culture. One microgram of total RNA was reverse transcribed and RT-PCR was performed. The GnRH mRNA levels were estimated by semi-quantitative RT-PCR as described in the Methods and Materials, and were normalized against β-actin (18 cycles) mRNA. Data were expressed as percent change relative to control and represent the mean +/- SE of four different experiments from four different patients. a, $p<0.05$, significantly different from the control day 1 cultures.

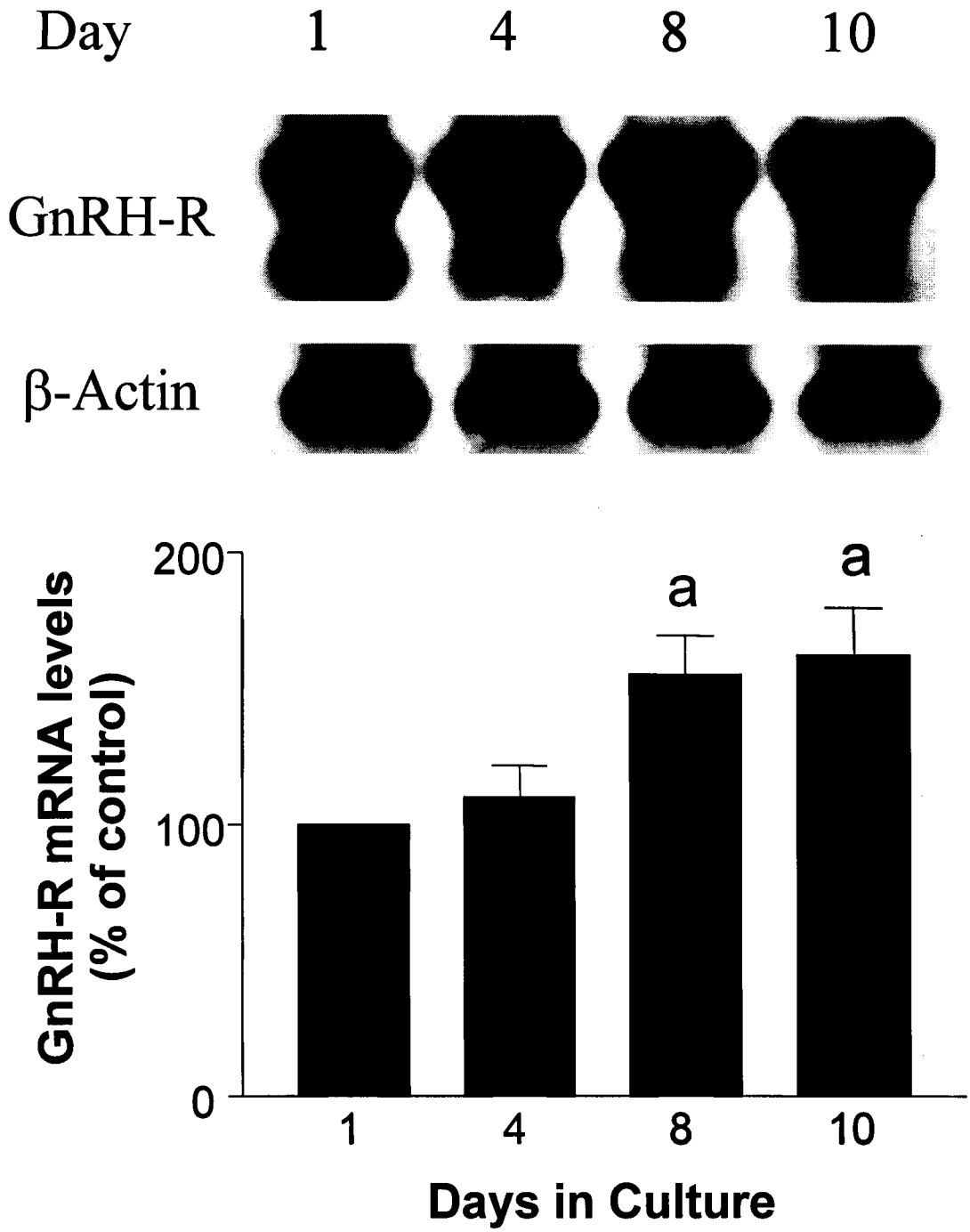


Figure 11. Changes in GnRH-R mRNA expression with spontaneous luteinization in culture. Total RNA was extracted from hGLCs (n=4) on day 1, 4, 8 and 10 in culture. One microgram of total RNA was reverse transcribed and RT-PCR was performed. GnRH-R mRNA levels were estimated by competitive RT-PCR as described in the Methods and Materials. The amount of GnRH-R transcript was calculated from the ratio of the native and mutant GnRH-R cDNA. Data were expressed as percent change relative to control and represent the mean +/- SE of four different experiments from four different patients. a, $p<0.05$, significantly different from the control day 1 cultures.

IV. Effects of estradiol on GnRH and GnRH-R expression in hGLCs

Human granulosa-luteal cells were treated in a dose- and time-dependent fashion to examine the effects of estradiol on GnRH and GnRH-R expression (Figs. 12, 13, 14, 15). As seen in Fig. 12, doses as low as 1nM decreased GnRH mRNA levels by 45% ($p<0.05$) relative to control values. A 70% decrease ($p<0.001$) in GnRH mRNA levels was observed with a dose of 100nM E2. Similarly, as seen in Fig. 13, estradiol had an inhibitory effect on GnRH-R mRNA levels. A significant decrease in GnRH-R mRNA levels (40%; $p<0.05$) was observed with a 1nM E2 treatment for 24h. A 65% decrease ($p<0.01$) in GnRH-R mRNA levels was observed at a 100nM E2 treatment. To further examine time-dependent changes (Figs. 14, 15), hGLCs were treated with 1nM E2 for 0, 6, 12, 24 and 48h followed by RNA extraction and RT-PCR quantitation. For each time point a control was included to take into account the changes during time in culture (data not shown). Relative changes of GnRH and GnRH-R mRNA levels were plotted as seen in Figs. 14, 15). Short-term treatment (6h) with E2 had no significant effect on GnRH mRNA levels. However, 24h treatment significantly decreased GnRH mRNA levels ($p<0.001$) compared to control. Long-term culture (48h) had no further effect on GnRH mRNA levels compared to 24h ($p>0.05$). Hence, maximal inhibition of GnRH mRNA was observed after 24h treatment with a 1nM dose of E2 (Fig 14). In contrast, a short-term treatment (6h) with E2 significantly increased GnRHR mRNA levels in hGLCs ($p<0.05$), followed by a significant decrease (45%; $p<0.05$) after a 24h treatment. Long-term treatment (48h) further decreased GnRH-R mRNA levels by 25% ($p<0.05$) when compared to the 24h treatment (Fig 15).

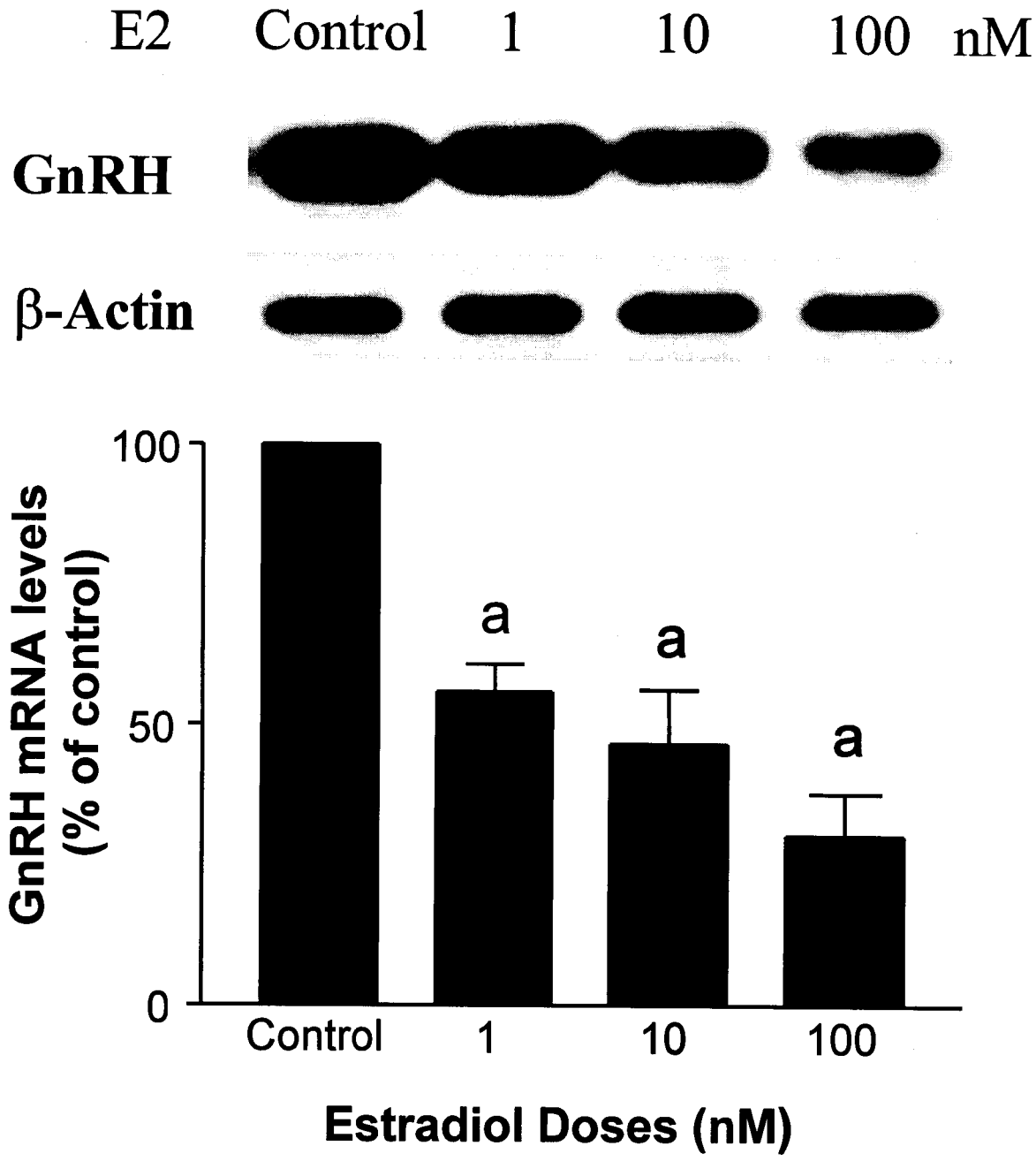


Figure 12. The effects of varying concentrations of 17β-estradiol on GnRH mRNA levels in cultured hGLCs. Cells were precultured for 4 days, and on day 5 were treated with various doses (0-100nM) of E2 for 24h. GnRH mRNA levels were estimated by semi-quantitative RT-PCR as described in the Methods and Materials, and were normalized against β-actin (18 cycles) mRNA. Data were expressed as percent change relative to control and represent the mean +/- SE of four different experiments from four different patients. a, $p<0.05$, significantly different from the control.

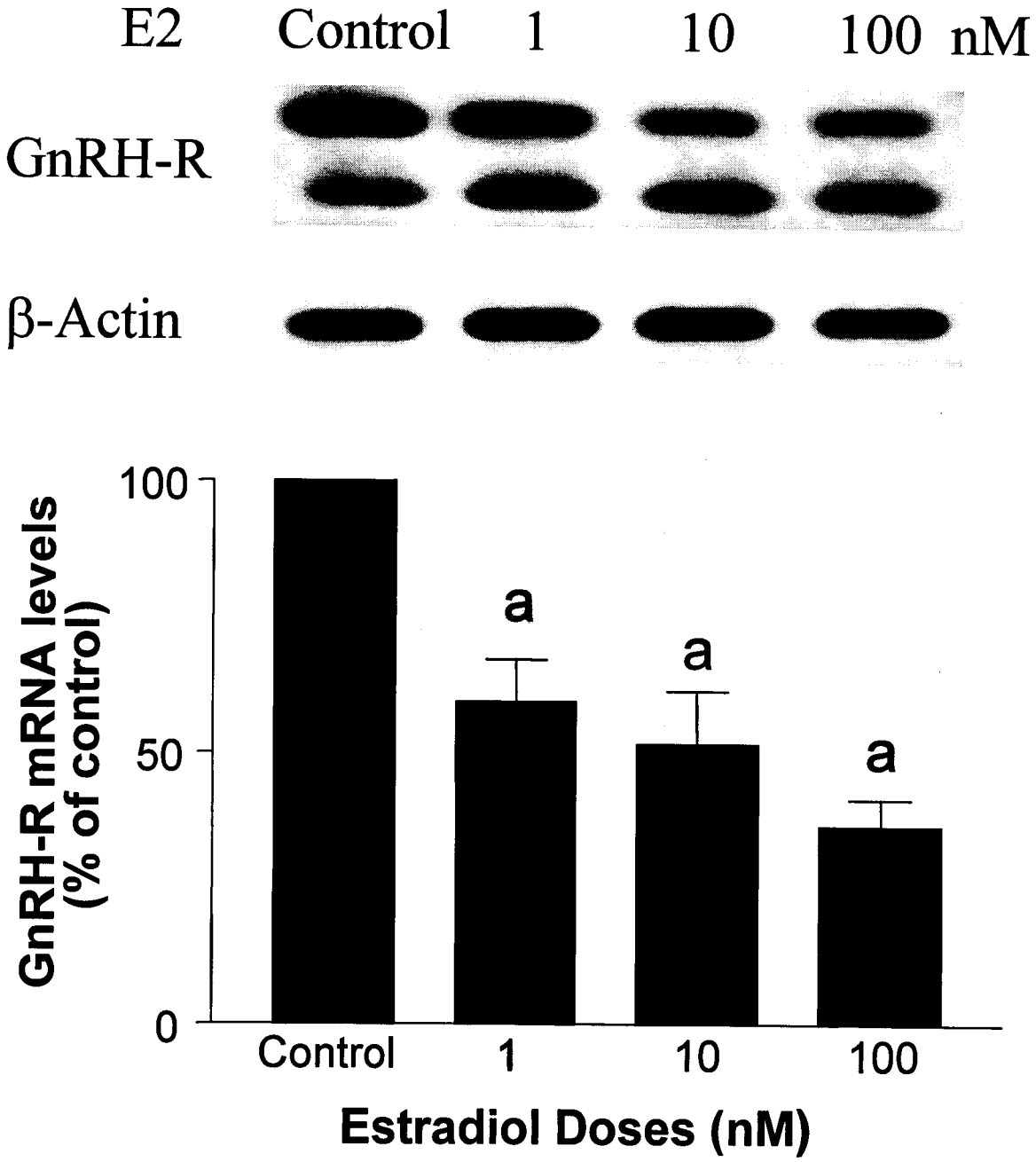


Figure 13. The effects of varying concentrations of 17β-estradiol on GnRH-R mRNA levels in cultured hGLCs. Cells were precultured for 4 days, and on day 5 were treated with various doses (0-100nM) of E2 for 24h. GnRH-R mRNA levels were estimated by competitive RT-PCR as described in the Methods and Materials. The amount of GnRH-R transcript was calculated from the ratio of the native and mutant GnRH-R cDNA. Data were expressed as percent change relative to control and represent the mean +/- SE of four different experiments from four different patients. a, $p<0.05$, significantly different from the control.

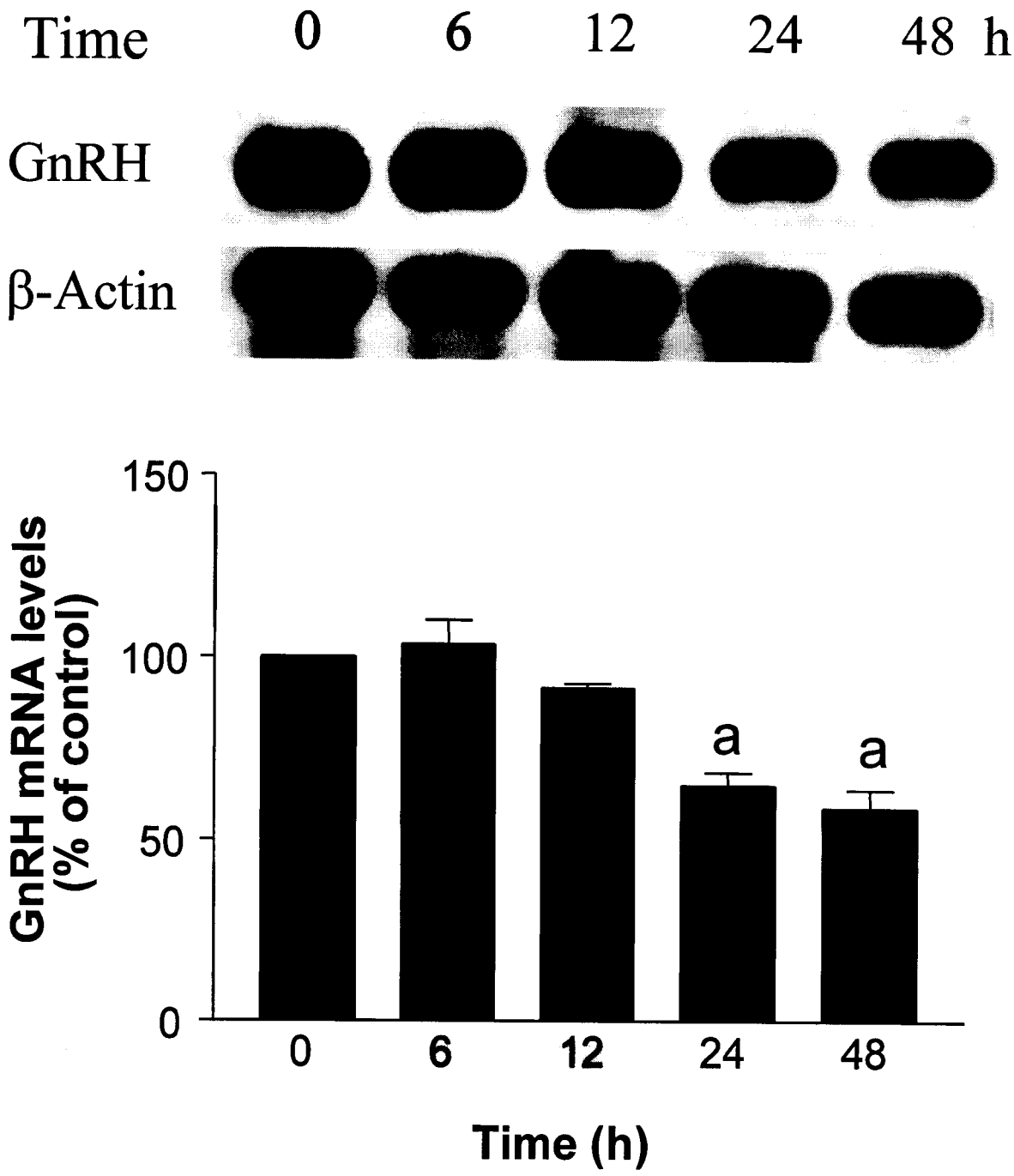


Figure 14. Time dependent effects of 17β-estradiol on GnRH mRNA levels in cultured hGLCs. Cells were precultured for 4 days, and on day 5 were treated with a 1 nM dose of E2 for 0, 6, 12, 24 and 48 h. GnRH mRNA levels were estimated by semi-quantitative RT-PCR as described in the Methods and Materials, and were normalized against β-actin (18 cycles) mRNA. Data was expressed as percent change relative to control and represent the mean +/- SE from four different experiments from four different patients. a, $p<0.05$, significantly different from the zero time point control.

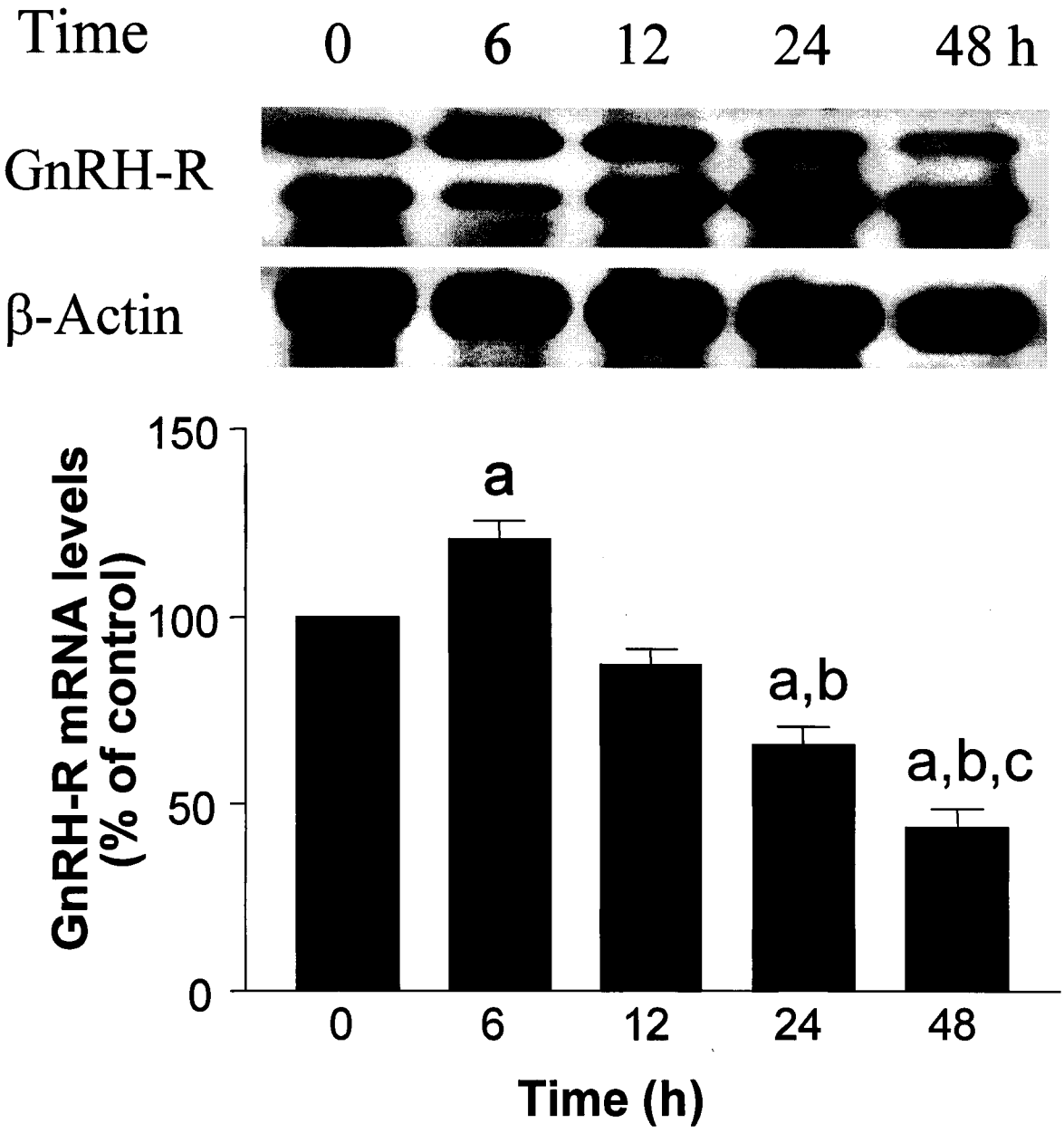


Figure 15. Time dependent effects of 17β-estradiol on GnRH-R mRNA levels in cultured hGLCs. Cells were precultured for 4 days, and on day 5 were treated with a 1 nM dose of E2 for 0, 6, 12, 24 and 48 h. GnRH-R mRNA levels were estimated by competitive RT-PCR as described in the Methods and Materials. The amount of GnRH-R transcript was calculated from the ratio of the native and mutant GnRH-R cDNA. Data were expressed as percent change relative to control and represent the mean +/- SE from four different experiments from four different patients. a, $p<0.05$, significantly different from the control; b, $p<0.05$, significantly different from the 6 h treatment group; c, $p<0.05$, significantly different from the 24 h treatment group.

V. Effects of co-treatment with estradiol and tamoxifen on GnRH and GnRH-R expression in hGLCs

To determine if the estradiol-induced regulation of GnRH and GnRH-R was a receptor-mediated event, hGLCs were treated with E2 (100nM) alone, or in combination with tamoxifen (10-100nM) for 24h. As seen in Figs. 16 and 17, E2 significantly decreased GnRH and GnRH-R mRNA levels ($p<0.001$). Tamoxifen alone had no significant effect on GnRH and GnRH-R mRNA levels (data not shown). Co-treatment with estradiol and tamoxifen reversed the response in a dose-dependent fashion. When hGLCs were treated with E2 and 10nM of tamoxifen, the mean GnRH and GnRH-R mRNA levels increased, but was not statistically significant compared to E2 treatment alone ($p>0.05$). However, when the cells were treated with equimolar doses of E2 and tamoxifen (100nM), the E2-induced down regulation of GnRH and GnRH-R was reversed. As depicted in Figs. 16 and 17, there was no statistical difference between the equimolar treatment group and the control group. However, there was a significant difference between the E2 treatment alone and equimolar treatment of E2 and tamoxifen ($p<0.001$).

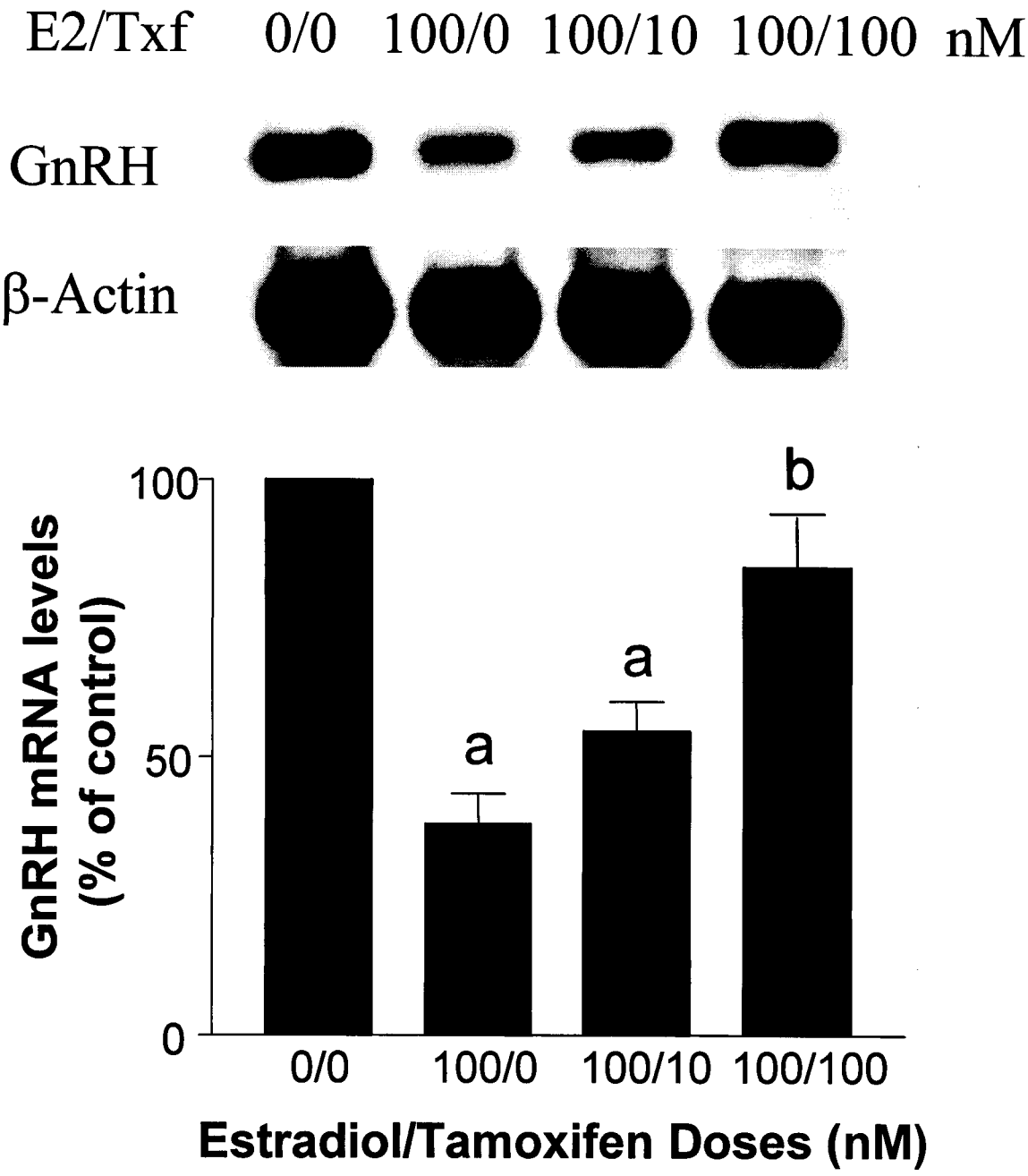


Figure 16. The effect of 17β-estradiol and tamoxifen co-treatment on GnRH mRNA levels in cultured hGLCs. Cells were precultured for 4 days, and on day 5 were treated with a 100nM dose of E2 in combination with varying doses of tamoxifen (0-100nM) for 24h. GnRH mRNA levels were estimated by semi-quantitative RT-PCR and were normalized against β-actin (18 cycles) mRNA. Data were expressed as percent change relative to control and represent the mean +/- SE of four different experiments from four different patients. a, $p<0.001$, significantly different from the control. b, $p<0.01$, significantly different from the E2 treatment.

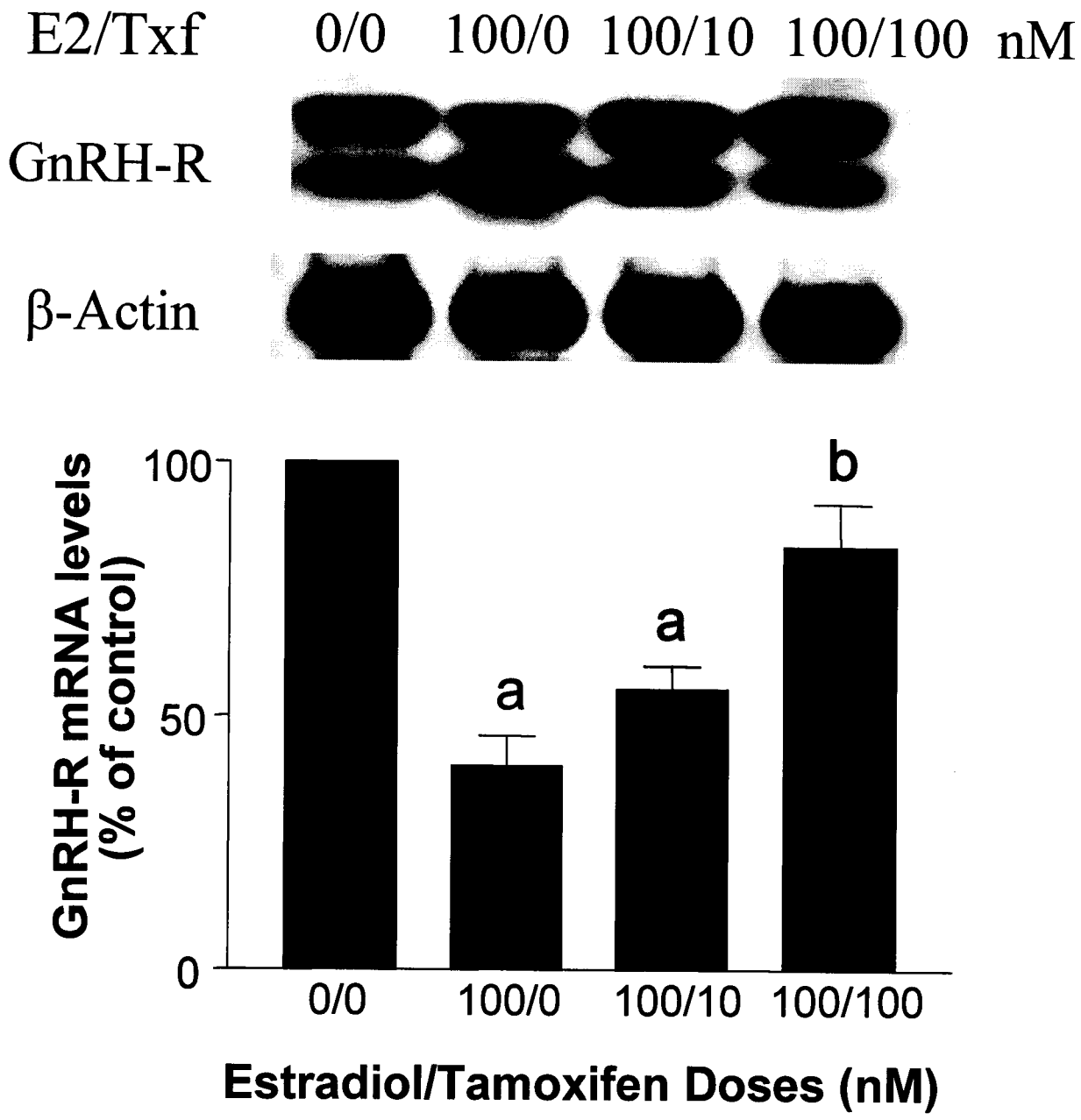


Figure 17. The effect of 17β-estradiol and tamoxifen co-treatment on GnRH-R mRNA in cultured hGLCs. Cells were precultured for 4 days, and on day 5 were treated with a 100nM dose of E2 in combination with varying doses of tamoxifen (0-100nM) for 24h. GnRH-R mRNA levels were estimated by competitive RT-PCR as described in the Methods and Materials. The amount of GnRH-R transcript was calculated from the ratio of the native and mutant GnRH-R cDNA. Data was expressed as percent change relative to control and represent the mean +/- SE of four different experiments from four different patients. a, $p<0.001$, significantly different from the control. b, $p<0.01$, significantly different from the E2 treatment.

VI. Effects of RU486 on GnRH and GnRH-R expression in hGLCs

Human granulosa-luteal cells were treated in a dose- and time-dependent fashion to examine the effects of RU486 on GnRH and GnRH-R expression (Figs. 18, 19, 20, 21). As seen in Fig. 18, RU486 had no significant effect on GnRH mRNA levels ($p>0.05$). Mean GnRH mRNA levels increased by 15% at a dose of 10000 nM of RU486. However, the increase was not statistically significant. In contrast, GnRH-R displayed a dose-dependent decrease with an increasing concentration of RU486 (Fig. 19). Although the mean GnRH-R mRNA levels decreased at a 1nM dose of RU486, there was no statistical difference when compared to the vehicle treated control ($p>0.05$). In contrast, a 30% and 50% decrease ($p<0.05$) in GnRH-R mRNA levels was observed at doses of 100 and 10000 nM of RU486, respectively. For the time course studies (Figs. 20, 21), hGLCs were treated with 10 μM RU486 for 0, 6, 12, 24, and 48h folowed by RNA extraction and RT-PCR quantitation. For each time point a control was included to take into account the changes during time in culture (data not shown). Relative changes of GnRH and GnRH-R mRNA levels were plotted as seen in Figs. 20 and 21. There was no significant change in GnRH mRNA levels ($p>0.05$) during the 48h treatment peroid with 10 μM RU486 (Fig. 20). However, GnRH-R mRNA levels exhibited a time-dependent inhibition with 10 μM RU486 (Fig. 21). A short term treatment (6h) had no significant effect on GnRH-R mRNA levels. Long-term treatment (24h) with RU486 significantly decreased GnRH-R expression by 50% ($p<0.05$), and this inhibition was sustained for 48h.

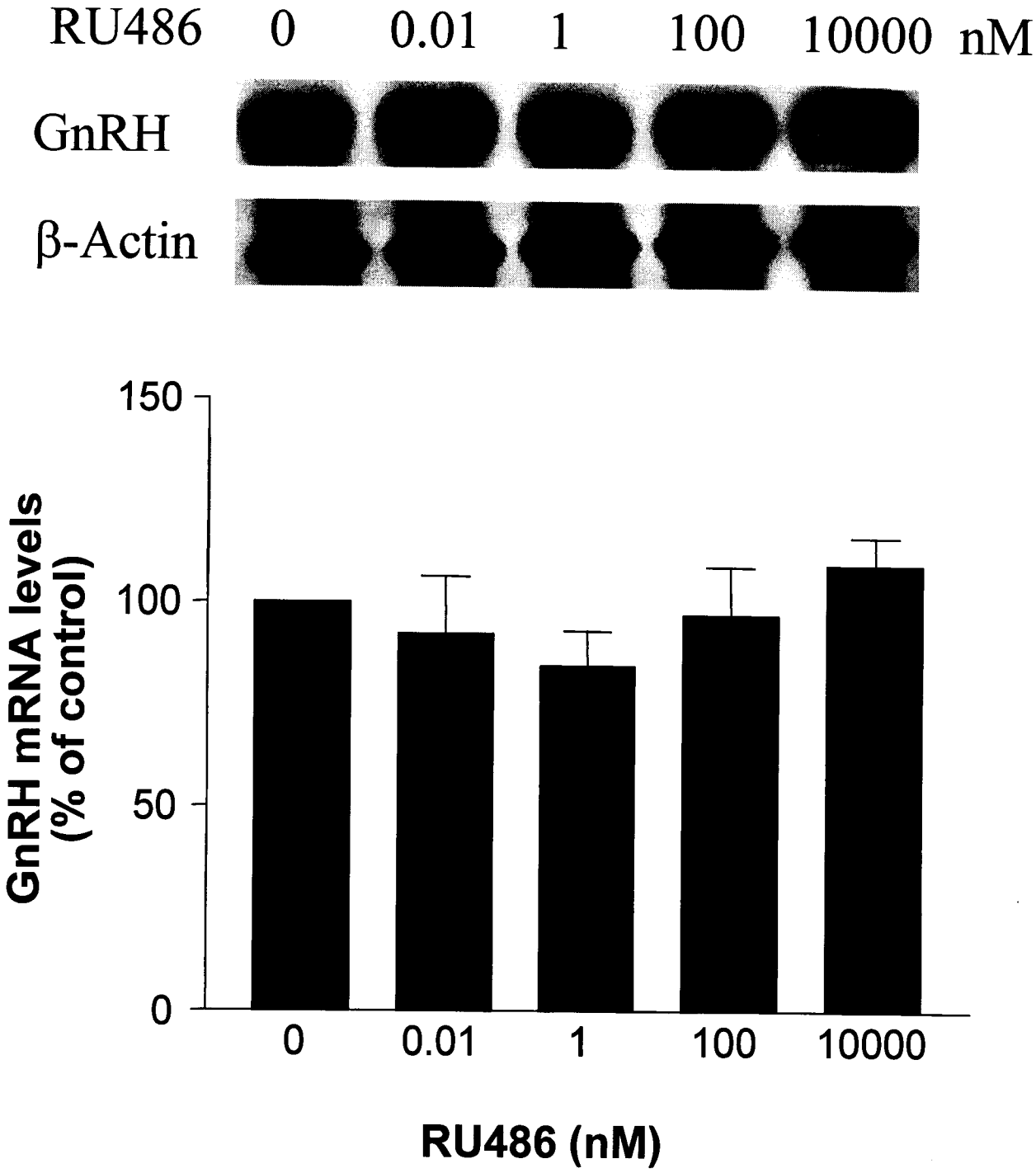


Figure 18. The effects of varying concentrations of RU486 on GnRH mRNA levels in cultured hGLCs. Cells were precultured for 4 days, and on day 5 were treated with various doses (0-10000 nM) of RU486 for 24h. GnRH mRNA levels were estimated by semi-quantitative RT-PCR, and were normalized against β-actin (18 cycles) mRNA. Data were expressed as percent change relative to control and represent the mean +/- SE of four different experiments from four different patients. a, $p<0.05$, significantly different from the control.

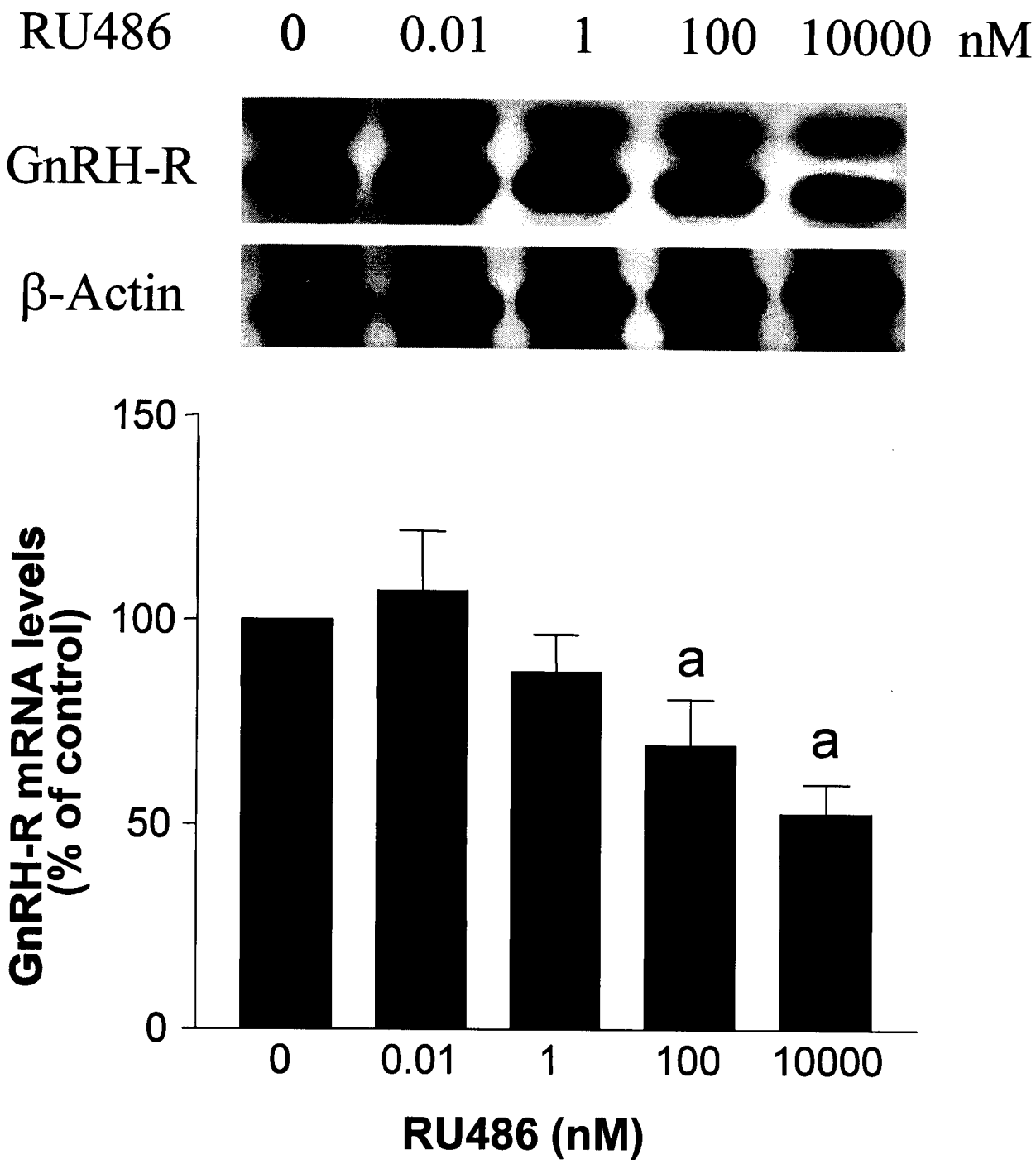


Figure 19. The effects of varying concentrations of RU486 on GnRH-R mRNA levels in cultured hGLCs. Cells were precultured for 4 days, and on day 5 were treated with various doses (0-10000 nM) of RU486 for 24h. GnRH-R mRNA levels were estimated by competitive RT-PCR as described in the Methods and Materials. The amount of GnRH-R transcript was calculated from the ratio of the native and mutant GnRH-R cDNA. Data were expressed as percent change relative to control and represent the mean +/- SE of four different experiments from four different patients. a, $p<0.05$, significantly different from the vehicle-treated controls.

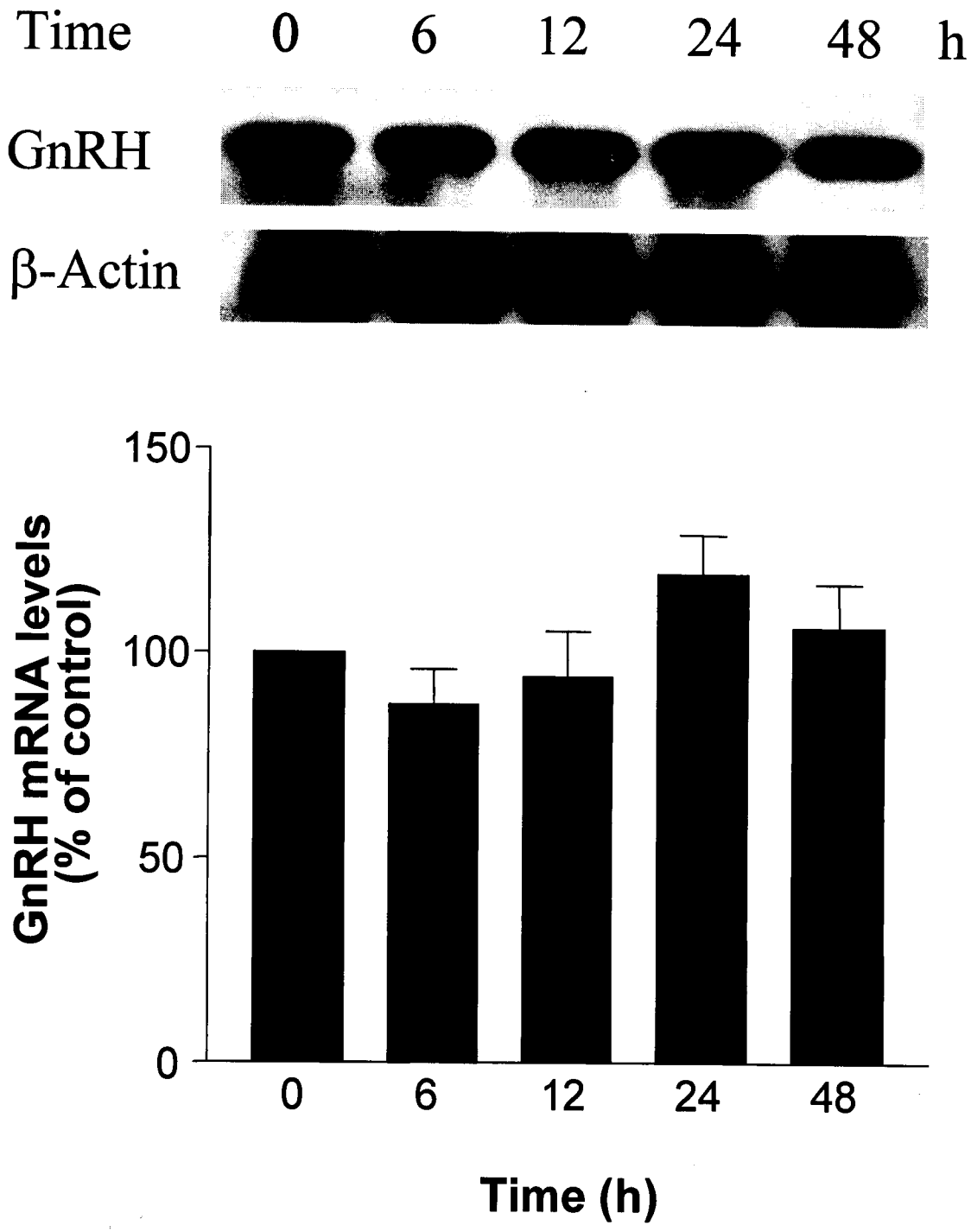


Figure 20. Time dependent effects of RU486 on GnRH mRNA levels in cultured hGLCs. Cells were precultured for 4 days, and on day 5 were treated with a 10 μM dose of RU486 for 0, 6, 12, 24 and 48 h. GnRH mRNA levels were estimated by semi-quantitative RT-PCR and were normalized against β-actin (18 cycles) mRNA. Data were expressed as percent change relative to control and represent the mean +/- SE from four different experiments from four different patients. a, $p<0.05$, significantly different from the time zero control.

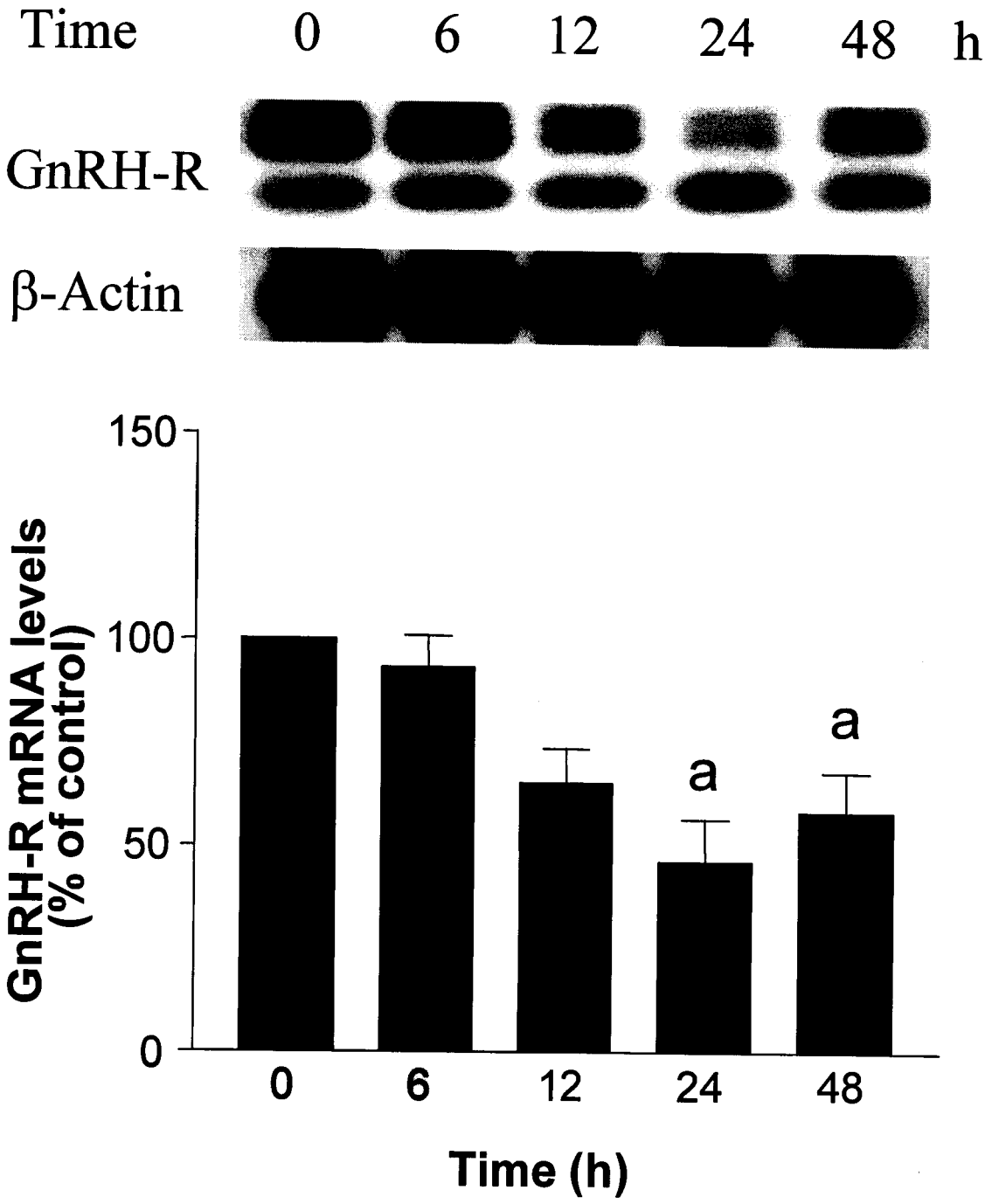


Figure 21. Time dependent effects of RU486 on GnRH-R mRNA levels in cultured hGLCs. Cells were precultured for 4 days, and on day 5 were treated with a 10 μM dose of RU486 for 0, 6, 12, 24 and 48 h. GnRH-R mRNA levels were estimated by competitive RT-PCR as described in the Methods and Materials. The amount of GnRH-R transcript was calculated from the ratio of the native and mutant GnRH-R cDNA. Data were expressed as percent change relative to control and represent the mean +/- SE from four different experiments from four different patients. a, $p<0.05$, significantly different from the time zero control.

VII. Expression and Function of GnRH-II cDNA from human ovarian cells

With the recent detection of a second form of GnRH in the primate brain (Lesheid et al, 1997), we used RT-PCR to examine the expression of GnRH-II in human ovarian cells (Fig. 22). As depicted in Fig. 22A, a pair of primers was designed based on the published human GnRH-II cDNA sequence (White et al., 1998). As shown if Fig. 22B, PCR amplification from hGLCs (lane 1), ovarian epithelial carcinoma cell line (OVCAR-3) (lane 2), primary ovarian cancer cells (lane 3), and ovarian surface epithelial cells (OSE) (lane 4) yielded the expected 225 bp fragment. Upon transfer of the PCR products to a nylon membrane and hybridization with a specific cDNA probe for GnRH-II a positive signal was detected in all lanes, confirming the identity of the PCR product (Fig 22B, lower panel). To ensure that that GnRH-II probe was specific and did not cross react with GnRH-I, a southern blot with GnRH-I was hybridized with the GnRH-II probe. No signal was detected after autoradiography, confirming that the GnRH-II probe was specific (data not show). The possibility of cross-contamination was ruled out, as no PCR product was detected in the negative control (Fig. 22B, lane 5). Since the primers were located in different exons, the amplified product was not due to genomic contamination, but rather the result of specific amplification of mRNA. Sequencing analysis revealed that the GnRH-II fragment from the ovarian cells was identical to the published sequence from the brain.

The functional role of GnRH-II in the ovary was examined using an established steroidogenesis assay. Human granulosa-luteal cells were treated with GnRHI or GnRHII alone, or in combination with antide for 24h. As seen in Fig. 23, GnRHI decreased progesterone secretion from hGLCs by 30% ($p<0.01$). Similarly, GnRHII

inhibited progesterone secretion into the culture media by 50% ($p<0.001$) compared to the vehicle treated controls. However, GnRHII inhibited progesterone secretion significantly more then GnRHI ($p<0.05$). Co-treatment with both analagues of GnRH and antide reversed the GnRH-induced inhibition of progesterone secretion from hGLCs.

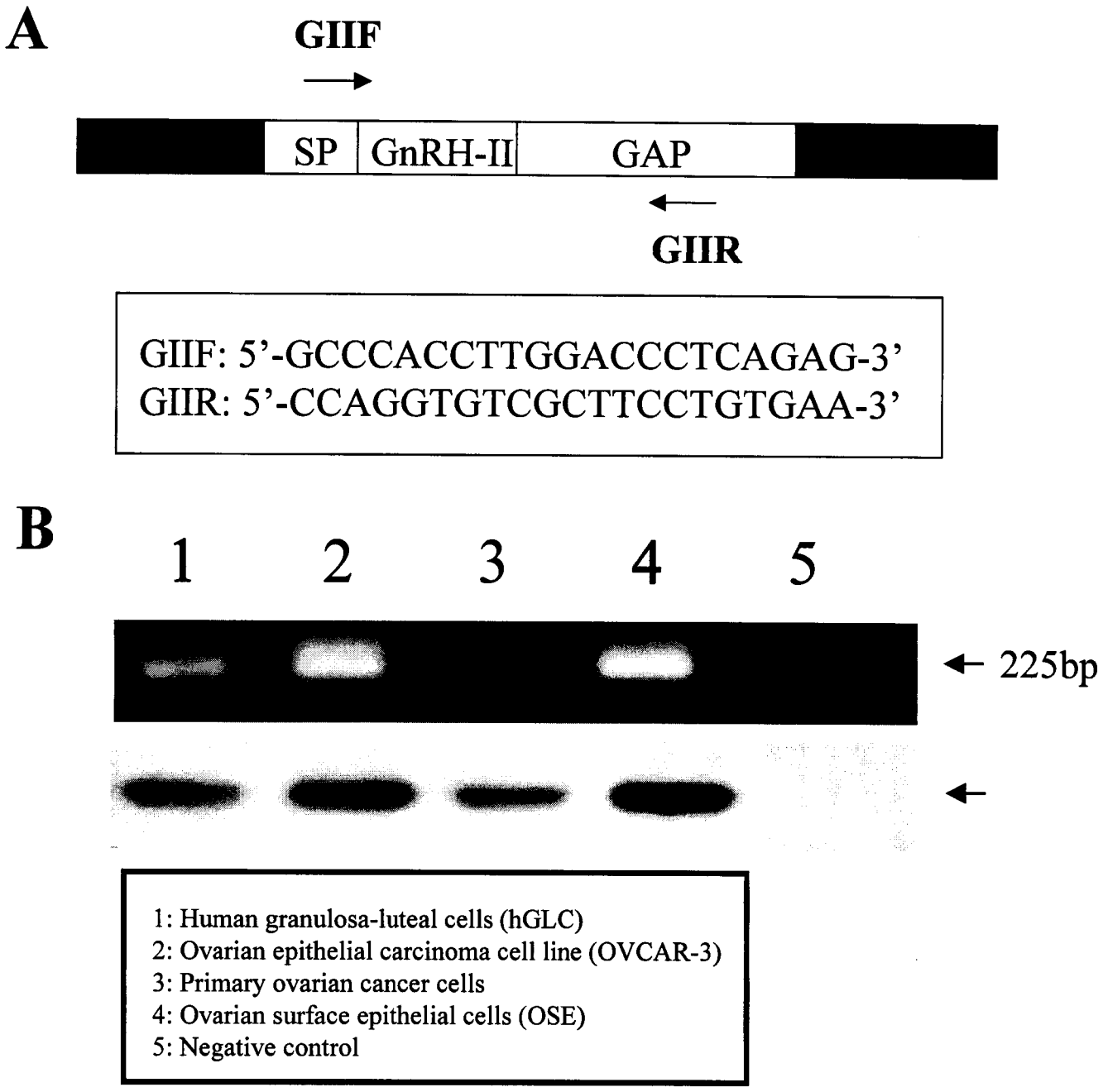


Figure 22. Expression of GnRH-II mRNA in human ovarian cells. (A). Schematic representation of the GnRH-II cDNA. The positions and sequences of the primers used for RT-PCR amplification of the coding region of GnRH-II are depicted. Primers were designed based on the published GnRH-II cDNA sequence from the human brain. SP, Signal peptide; GAP, GnRH associated peptide. (B). Total RNA was extracted from hGLCs (lane 1), ovarian cancer cell line-OVCAR-3 (lane 2), primary ovarian cancer cells (lane 3), and ovarian surface epithelial cells (lane 4). First strand cDNA was synthesized and subjected to PCR amplification for 35 cycles. A negative control without cDNA template was used to control for PCR contamination (lane 5). The PCR products were resolved on a 1% agarose gel stained with ethidium bromide. The expected 225-bp fragment was visualized under ultraviolet light, and was confirmed to be GnRH-II by Southern blot analysis (lower panel).

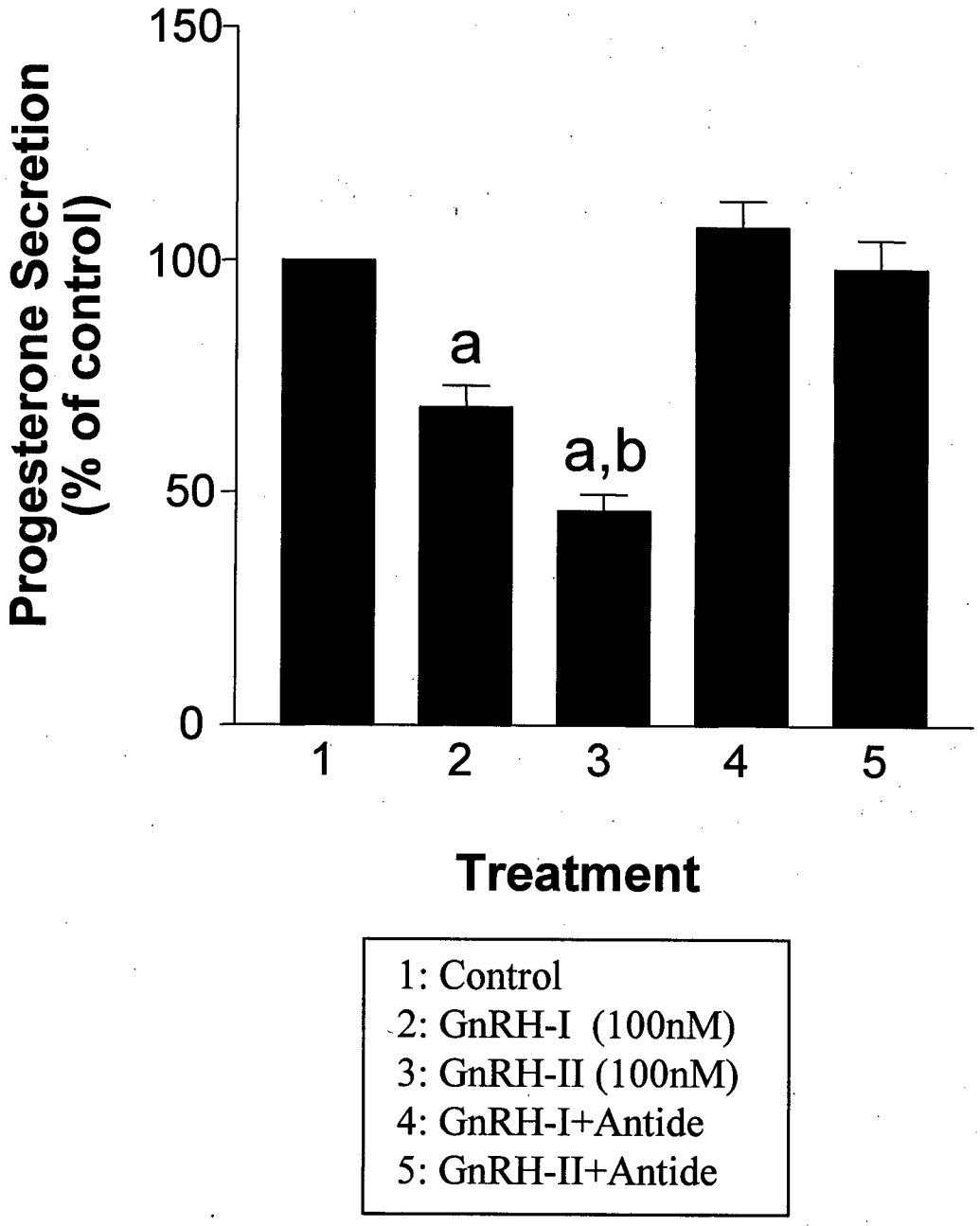


Figure 23. The effect of GnRH-I and GnRH-II on progesterone secretion from cultured hGLCs. Human GCs were seeded at a density of 1.5 X 10^5 cells in 35mm dishes and precultured for 4 days. On day 5 the cells were treated with GnRH-I or GnRH-II (100nM) alone or in combination with antide (1 μM) for 24h. Vehicle treated cultures acted as controls. Subsequently the media was collected and assayed for progesterone content and the cells were lysed to measure total protein. Progesterone content was standardized to total protein content. Data were expressed as a percent change relative to control and represent the mean +/- SE from four different experiments from four different patients. a, $p< 0.05$, significantly different from the vehicle treated control; b, $p<0.05$, significantly different from the GnRH-I treatment group.

G. DISCUSSION

Luteinization is a complex differentiation process that is under the influence of many gonadal factors such as gonadotropins, steroids and growth factors. However the exact regulatory mechanisms underlying luteinization and regression remain unknown. GnRH has been implicated as an autocrine/paracrine regulator of luteal function and luteolysis (Valbuena et al., 1997). Since GnRH actions are mediated through a G-protein coupled receptor, the expression of the GnRH-R is crucial for GnRH actions. Previous studies demonstrated that cultured hGLCs express the components of the GnRH system (Tureck et al., 1982; Peng et al., 1994), and provide an excellent model to study the autocrine/paracrine function and regulation of GnRH in the ovary. The use of primary cell culture *in vitro* model systems has several advantages. First, the isolation procedure requires no proteolytic or modifying enzymes that may change responses to physiological stimuli. In addition, it has been shown that primary cultures of granulosa cells, unlike other ovarian cell lines, retain responsivness to hormonal stimuli that resemble *in vivo* conditions (Hsueh et al., 1981). The *in vitro* culture system employed in this study eliminates the effects of confounding factors from the hypothalamo-pituitary axis. Furthermore, since the hGLCs are cultured in the absence of an androgen substrate, the exogenous effects of estradiol are not influenced by the endogenous production. This idea is further corroborated in this study as tamoxifen treatment alone had no significant effect of GnRH and GnRH-R gene expression (data not shown). However, since hGLCs come from patients in the IVF program, the locus and cause of infertility is unknown. Thus, changes in gene expression in response to exogenous factors may not represent the normal physiological situations. Nevertheless, this culture system is commonly used to

study ovarian endocrinology in the human (Clayton et al., 1979 Peng et al., 1994; Vaannanen et al., 1997).

While expression of the GnRH-R from the human ovary has been previously reported (Peng et al., 1994), molecular characterization of the ovarian transcript equivalent to the full length GnRH-R in the pituitary has not been documented. Using radioreceptor assays, many reports have suggested that the binding affinity for the GnRH-R in extrapituitary tissues is 10-10000 fold lower compared to the pituitary (Jones et al., 1980; Kakar et al., 1994). Studies indicate that the molecular structure of hypothalamic GnRH and extrapituitary GnRH are identical (Peng et al., 1994; Boyle et al., 1998). Thus based on the differential binding affinities, it has been suggested that the molecular structure of the pituitary GnRH-R may be distinct from the extrapitutiary receptor. Furthermore, mutations in the GnRH-R have been documented in patients with hypogonadotropic hypogonadism (Layman et al., 1998; Cohen et al., 1999). The present study isolated the entire coding region of the GnRH-R from hGLCs by RT-PCR amplification. Sequence analysis of the 1kb fragment revealed that the ovarian GnRH-R is identical to the pituitary cDNA sequence (Kakar et al., 1992). Similar results were also obtained when the GnRH-R in the placenta, ovarian cancer cells and the prostate were compared to their pituitary counterparts (Kakar et al., 1994; Moumni et al., 1994; Boyle et al., 1998). These findings are surprising as the binding studies demonstrate significant differences amongst the pituitary and extrapituitary receptors. Accordingly, several hypotheses have been put forth to explain such differences. Several groups have documented that the extrapituitary tissues possess high affinity binding sites (in contrast to low affinity binding sites) similar to those found in the pituitary (Currie et al., 1981;

Fekete et al., 1989; Yano et al; 1994), and thus, it is not surprising that the molecular structure of the receptor is identical amongst the tissues in question. Alternatively, others suggest that although the mRNA sequence for the GnRH-R is identical in the various tissues, the transcripts may differ in their post-translational modifications, including phosphorylation and glycoslyation sites which may be responsible for the differing binding affinities (Moumni et al., 1994; Boyle et al., 1998). Currie et al. have suggested that the differences in binding affinities may be due to different forms of GnRH present in different tissues. This hypothesis is further substantiated with the recent detection of GnRH-II in humans (White et al., 1998). Finally, as in some lower vertebrates including fish, it has been suggested that there may be more then one form of the GnRH-R present within a single species (Boyle et al., 1998; Illing et al., 1999).

Results from the present study indicate that GnRH and its receptor mRNA levels significantly increase with spontaneous luteinization *in vitro*. Changes in GnRH mRNA levels are also seen during postnatal development and through puberty. Increases in GnRH mRNA levels during development are hypothesized to play a crucial role in regulating the onset of puberty as premature administration of GnRH induced precocious puberty in immature animals (Wildt et al., 1980; Gore et al., 1999). Although the mechanism of increased GnRH and GnRH-R mRNA in the ovary during luteinization remains unknown, in the mouse changes in GnRH mRNA levels during development are attributed to gene transcription and altered mRNA stability (Gore et al., 1999). Similar results were also obtained around the peripubertal peroid in female rats (Sisk et al., 1996). The increase in GnRH and its receptor mRNA levels in our culture system may be due to the endogenous production of ovarian factors. Functionally, the dynamic

changes in GnRH and its receptor mRNA during luteinization suggest an important role autocrine/paracrine role for GnRH during the luteal phase of menstrual cycle.

As gonadal steroids are key regulators of reproduction, the effects of estradiol and progesterone on GnRH and its receptor gene expression at the hypothalamo-pituitary level are well-documented. For example, during the rat estrous cycle, GnRH mRNA levels in the anterior hypothalamus were inversely correlated to plasma estrogen levels, providing evidence that E2 may directly or indirectly inhibit hypothalamic GnRH mRNA levels (Zoeller and Young, 1988). However, other groups have documented that E2 increases GnRH gene expression in the rat (Roberts e al., 1989; Park et al., 1990), which may contribute to the gonadotropin surge prior to ovulation. The discrepancies observed between different groups may be due to the anatomical regions analyzed, time points during the estrous cycle and/or differences in sensitivities of techniques. No information is available on the role of steroids in regulating GnRH and its receptor in the ovary, especially in the human. In this study we demonstrate for the first time that estradiol negatively regulates GnRH mRNA in cultured hGLCs, and significant effects were seen at physiological doses of 1 nM after 24 h treatment.

Like GnRH, regulation of GnRH-R by E2 in the pituitary is well documented. In the sheep, mouse and rat, estradiol increased GnRH-R mRNA levels (Gregg et al., 1990; McArdle et al., 1992; Quinones-Jenab et al., 1996; Sakurai et al., 1997). However, other groups have shown that E2 negatively regulates GnRH-R mRNA levels in the rat pituitary gland (Kakar et al., 1994). Differences in steroid-induced regulation of GnRH-R also display tissue specificity. For example, in the rat hippocampus, E2 significantly decreased GnRH-R levels (Badr et al., 1987; Jennes et al., 1995). Taken together, these

data suggest that the GnRH-R is differentially regulated depending on the tissue and species. Our study demonstrates that GnRH-R mRNA displays a biphasic regulatory pattern. Short term treatments with E2 (6h) resulted in an increase in GnRH-R mRNA levels, whereas long term treatments resulted in a significant decrease in GnRH-R mRNA levels.

The exact mechanism by which E2 regulates GnRH and its receptor mRNAs in the ovary remains uncertain. Others have documented that the ovary expresses estrogen receptor α and β (Misao R et al., 1999), and our studies confirm that the E2 mediated regulation of GnRH and GnRH-R is a receptor-mediated event as co-treatment with tamoxifen blocked the E2 induced decrease in GnRH and GnRH-R. Similar results were also obtained in the pituitary (Gregg et al., 1990). After binding to its receptor, E2 may modulate GnRH and GnRH-R directly or indirectly. Analysis of the human GnRH and GnRH-R promoter region reveals no consensus sequence for an estrogen response element (ERE) (Fan et al., 1995; Radovick et al., 1991). However, E2 can directly modulate transcription of the GnRH gene (Radovick et al., 1991; Dong et al., 1996), and preliminary evidence from our laboratory suggests that E2 may modulate GnRH-R promoter activity. Interestingly, previous studies have shown that E2 decreased GnRH promoter activity in placental cells (Dong et al., 1992), providing further support for the E2 induced down regulation of GnRH mRNA in the extrapituitary hGLCs. Alternatively, E2 may act through indirect pathways to regulate GnRH and its receptor in hGLCs. It has been demonstrated that E2 can mobilize intracellular Ca^{2+} in chicken granulosa cells (Morley et al., 1992), which may lead to activation of the protein kinase C (PKC) pathway. There is evidence that activation of the PKC pathway can modulate GnRH-R

gene expression (Conn et al., 1984). The present study indicates that the E2-induced down regulation of GnRH and its receptor is a receptor-mediated event.

Like estradiol, the role of progesterone in regulating GnRH and its receptor at the level of the hypothalamus and pituitary is well documented. Previous studies demonstrate that progesterone down regulates hypothalamic GnRH mRNA expression in rats (Toranzo et al., 1989). In rat GT1-7 hypothalamic cells, the direct binding of the progesterone receptor on a nonconsensus DNA sequence repressed GnRH expression (Kepa et al., 1996). Similarily, in ovine progesterone decreased GnRH sensitivity and binding in pituitary gonadotrope cultures, and this effect was mediated through the progesterone-induced decrease in GnRH-R number (Batra and Miller, 1985; Laws et al., 1990). The present study investigated the role of progesterone in regulating ovarian GnRH and its receptor. Preliminary studies using direct progesterone administration in hGLCs revealed contradictory and inconclusive data (data not shown). Since hGLCs possess all the enzymes for *de novo* synthesis of progesterone (Hseuh et al., 1981), the observed discrepancies may have been due to the variations in endogenous progesterone production from different patients. Accordingly, we used RU486, a progesterone antagonist, to indirectly examine the effects of progesterone on GnRH and its receptor mRNA expression in hGLCs. Clinically, RU486 is commonly used to treat uterine leiomyomata, endometriosis, and can cause first trimester abortions. (Morales et al., 1996; Murray and Muse, 1996). Results from the present study indicated that RU486 had no significant effect on GnRH mRNA levels. However, there was a dose- and time-dependent decrease in GnRH-R mRNA levels in response to this progesterone antagonist, confirming that progesterone increases GnRH-R mRNA in cultured hGLCs. Although

the exact regulatory mechanism of the progesterone-induced regulation of GnRH-R is not known, preliminary evidence from our laboratory suggests that RU486 decreased GnRH-R promotor activity and GnRH-R mRNA in placental cells (Cheng et al., unpublished data). These results provide further support for the RU486 induced down-regulation of GnRH-R mRNA in the extrapituitary hGLCs. The differences seen between the progesterone-induced regulation of GnRH-R in the ovine pituitary and hGLCs may be attributed to species and tissue specific regulatory mechanisms. This idea is further supported by the lack of progesterone-induced regulation of GnRH-R in the rat hippocampus (Badr et al., 1988).

It is important to note that although the current study examined the effects of estradiol and progesterone on GnRH and its receptor expression in hGLCs, it is possible that estradiol and progesterone act synergistically to modulate gene expression in the ovary. Indeed, E2 has been shown to regulate the progesterone receptor in cultured rat gonadotrope cells, suggesting that estradiol effects may be mediated by modulating progesterone actions (Turgeon et al., 1999). For example, combination of E2 and P4 treatments in ovine pituitary cultures resulted in the greatest modulation of GnRH-R expression (Gregg et al., 1990; Sakurai et al., 1997). Similar results were also obtained in the rat hippocampus (Badr et al., 1988). In contrast, progesterone has been shown to block specific estradiol induced signals in sheep pituitaries (Harris et al., 1999). As a result, further studies are required to analyze the interaction between GnRH and gonadal steroids in the human ovary.

Activation of the GnRH-R in extrapituitary tissues, in contrast to pituitary cells, may activate the programmed cell death pathways by interacting with the Fas/Fas Ligand

pathway (Murdoch, 1995). It has been demonstrated that the Fas L is capable of inducing apoptotic cell death in rat luteal cells and hGLCs (Quirk et al., 1995; Roughton et al., 1999). In rat granulosa cells, GnRH directly induced apoptosis and may be an important factor in regulating follicular atresia (Billing et al., 1994). Sridaran et al (1998) have reported that GnRH decreased serum progesterone levels and induced apoptotic cell death in the corpus luteum (CL) of early pregnancy rats (Sridaran et al., 1998). However, since this was an *in vivo* study, the direct effects of GnRH on the ovary cannot be dissected from the influence of the hypothalamo-pituitary axis. Recently, GnRH has been shown to directly increase the incidence of apoptosis in hGLCs (Zhao et al., 2000). Furthermore, previous reports have implicated GnRH as a luteolytic factor. For example, LH/hCG decreased GnRH-R expression in the rat and human ovary (Peng et al., 1994; Olofsson et al., 1995). Human CG is an important rescue factor for the maintenance of the corpus luteum during the early stages of pregnancy. Hence, the down regulation of GnRH-R by LH/hCG may be involved in the maintenance of the corpus luteum during pregnancy. Our study demonstrates an increase in GnRH and its receptor mRNAs during spontaneous luteinization *in vitro*, which suggests that GnRH in combination with other factors, may play an important role in corpus luteum regression. Indeed, it has been suggested that GnRH potentiates the antigonadotropic effects of PGF2α in hGLCs (Vaannanen et al., 1997). GnRH has been shown to inhibit the gonadotropin-induced response in hGLCs, which may be clinically linked to the decreased quality follicular development seen in patients treated with GnRH agonists for ovulation induction (Furger et al., 1996). This idea is further corroborated by the decreased steroidogenic potential, a

biochemical marker of functional luteolysis (Duncan et al., 1998), with GnRH treatment in hGLCs (Clayton et al., 1979; Tureck et al., 1982; Peng et al., 1994).

While appreciable research has focused on the role of growth factors in the autocrine/paracrine modulation of ovarian functions, the role of gonadal steroids in the ovary during the luteal phase is poorly understood. Nevertheless, the expression of functional estrogen and progesterone receptors in the hGLCs and the corpus luteum suggest a possible autocrine/paracrine role of gonadal steroids in the ovary (Chaffkin et al., 1993; Brandenberger et al., 1998; Misao et al., 1999). This idea is further supported by the E2-induced increase in progesterone production during the early luteal phase in rabbits and humans (Holt and Schreiber, 1985; Endo et al., 1998; Wuttke et al., 1998). Furthermore, deprivation of E2 in the rabbit corpus luteum resulted in a time-dependent induction of apoptosis (Goodman et al., 1998). Thus, since GnRH actions have been linked to induction of apoptosis in the ovary, the E2-induced down regulation of GnRH and its receptor in hGLCs may play an important role in the maintenance of the corpus luteum. Moreover, progesterone inhibited hGLCs proliferation and differentiation (Chaffkin et al., 1992; Chaffkin et al., 1993). Similarily, GnRH inhibited cell growth in the ovarian surface epithelium and induced apoptosis in ovarian cancer cells (Kang et al., 2000; Kang et al., submitted). Thus, the progesterone-induced increase in GnRH-R expression in hGLCs seen in our study may be one of the important factors regulating the demise of the corpus luteum during the mid- to late luteal phase. However, further studies are required to substantiate this hypothesis.

Clinically, since a GnRH regimen is a common practice in ART, the direct ovarian effects of GnRH agonists on follicle development and quality have been raised

(Lefevre et al., 1991; Testart J et al., 1993). Thus, understanding the dynamics of the intrinsic ovarian GnRH axis may play an important role in the discernment of ART outcomes.

In addition to the regulation studies on GnRH and its receptor, the current study examined the expression and function of a second form of GnRH in the human ovary. With the duplications and rearrangments of the GnRH gene throughout evolution, individual species may have more then one form of GnRH. With the recent characterization of a second form of GnRH from the human brain (White et al., 1998), understanding the significance of multiple forms of GnRH in vertebrates has become a major concern (Carolsfeld et al., 2000). We have demonstrated that the ovarian surface epithelium, ovarian cancer cells and hGLCs express a second form of GnRH, analogous to the chicken II form. Interestingly, among all the different forms of GnRH, only GnRH-II is identical in all species. This suggests that maybe GnRH-II has different functions compared to GnRH-I. Indeed, GnRH has been implicated in non-reproductive functions, including neuromodulatory and immune actions (Troskie et al., 1997; Chen et al., 1999). Functionally, like GnRH-I, GnRH-II inhibited progesterone secretion from cultured hGLCs. However, GnRH-II induced a more marked decrease in P4 secretion compared to GnRH-I. These results are not surprising as previous reports suggest that the GnRH-R binds GnRH-II more effectively then GnRH-I (King and Millar, 1991). Furthermore, with the existence of two or more forms of GnRH within the same species, the question of different receptor types for the variable forms of GnRH has been raised (Illing et al., 1999). In humans, binding studies from extrapituitary tissues reveal contradicitory results. Some groups suggest that the extrapituitary tissues possess a low

affinity GnRH-R, while others suggest a high affinity receptor. Similar results were observed in goldfish, where a second GnRH-R with differential tissue distribution and ligand selectivity has been characterized. Although only one form of GnRH-R has been cloned from mammals, the expression of a second form of GnRH in humans suggests that there may be more then one receptor. Recent evidence indicates that a type II GnRH-R gene is present in humans and is expressed in a wide variety of tissues. However, the detected transcripts were in the antisense orientation, indicating that no functional protein would translated from the type II GnRH-R transcript in humans (Millar et al., 1999). Thus to date, only one GnRH-R exists in humans. Further studies on binding affinities and tissue distribution of GnRH-II will help clarify the role of this highly conserved peptide in humans.

H. SUMMARY

In summary, we have isolated the ovarian GnRH-R transcript that is equivalent to the full-length GnRH-R cDNA from the pituitary in cultured hGLCs, and determined that the cDNA sequence is identical to its counterpart in the pituitary gland. In addition, we observed an increase in GnRH and GnRH-R expression with spontaneous luteinization in culture, suggesting that GnRH may play a role in controlling corpus luteum function. Furthermore, estradiol induced a down regulation of GnRH and its receptor mRNA in hGLCs, while RU486 decreased GnRH-R mRNA expression. Our findings demonstrate for the first time, that gonadal steroids can regulate the expression of GnRH and GnRH-R in the human ovary. Since GnRH actions have been linked to induction of apoptosis, and luteolysis has been demonstrated to occur via an apoptotic mechanism, the dynamic balance between estradiol and progesterone during the luteal phase may play a role in regulating the fate of the corpus luteum. Moreover, we demonstrate that human ovarian cells express a second form of GnRH, distinct from the traditional mammalian form. Functionally, GnRH-II induced a decrease in steroidogenic potential in hGLCs. In conclusion, the dynamic regulation of GnRH and its receptor, coupled with the expression and function of GnRH-II in the ovary, strengthen the notion that GnRH acts as an autocrine/paracrine modulator of human ovarian functions.

I. FUTURE STUDIES

Although the present study examined the effects of estradiol and progesterone and GnRH and its receptor gene expression in hGLCs, the interaction between estradiol and progesterone must be elucidated to better understand the dynamics of the intrinisic ovarian GnRH axis. Furthermore, transfection studies to characterized the regulatory elements on the promotor of the GnRH and its receptor gene will prove valuable in understanding the mechanisms that regulate their expression in the ovary. These studies are currently in progress in Dr. Leung's laboratory. Functionally, the role of estradiol and progesterone in modulating GnRH-induced apoptosis in the ovary are required to substantiate the hypothesis put forth in this study.

With the expression of GnRH-II in the humans, we must re-evaluate the notion that reproduction is control by one form of GnRH. The possiblity of a second GnRH-R in humans must be explored to understand the functional signficiance of multiple forms of GnRH. Future studies involving tissue specific regulation and distribution of GnRH-II will clarify the functional role that this highly conserved peptide plays in human reproduction.

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