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Potential role of gonadotrophin-releasing hormone (GnRH)-I and GnRH-II in the ovary and ovarian cancer

S K Kang¹, K-C Choi, H-S Yang² and P C K Leung

Department of Obstetrics and Gynaecology, University of British Columbia, 2H-30, 4490 Oak Street, Vancouver, British Columbia V6H 3V5, Canada,

¹Department of Theriogenology and Biotechnology, College of Veterinary Medicine, Seoul National University, Seoul, Korea

²Department of Obstetrics and Gynaecology, College of Medicine, Dongguk University Kyongju, Korea

(Requests for offprints should be addressed to P C K Leung; Email: peleung@interchange.ubc.ca)

Abstract

Gonadotrophin-releasing hormone (GnRH) functions as a key neuroendocrine regulator of the hypothalamic–pituitary–gonadal axis. In addition to the hypothalamus and pituitary gland, GnRH and its receptor have been detected in other reproductive tissues including the gonads, placenta and tumours arising from these tissues. Recently, a second form of GnRH (GnRH-II) and type II GnRH receptor have been found in normal ovarian surface epithelium and neoplastic counterparts. The two types of GnRH may play an important role as an autocrine/paracrine regulator of reproductive functions and ovarian tumour growth. In this review, the distribution and potential roles of GnRH-I/-II and their GnRH receptors in the ovarian cells and ovarian cancer will be discussed.

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Molecular structure of gonadotrophin-releasing hormones (GnRHs) and GnRH receptors (GnRHRs)

Cloning and molecular structure of GnRHs

GnRH was first isolated and sequenced from mammals (Schally 1999), and now 13 distinct forms of GnRH have been found in various species (Carolsfeld *et al.* 2000). In fish, amphibians, reptiles and birds, there are two or more forms of GnRH within the brain of single species (Sherwood *et al.* 1993, White *et al.* 1995). The primate brain was thought to contain only the GnRH known as mammalian GnRH (mGnRH, now here designated as GnRH-I). However, the two forms of GnRH, which were similar to mGnRH and chicken GnRH-II (cGnRH-II), have been discovered in brain extracts from adult stump-tail and rhesus monkeys (Lescheid *et al.* 1997). Subsequently, a gene encoding the second form of GnRH has been cloned in the human (White *et al.* 1998). The GnRH genes consist of four exons and three introns and exist as part of a larger precursor gene as demonstrated in Fig. 1 (Sherwood *et al.* 1993, White *et al.* 1998). The precursor cDNA consists of GnRH that is extended at the N ter-

minus by a signal peptide and at the C terminus by a Gly-Lys-Arg sequence, characteristic of an enzymatic amidation and precursor processing site, followed by a GnRH-associated peptide (GAP). Distinct distributions of neurons containing both forms of GnRH in the primate brain have been demonstrated. The majority of GnRH-I-synthesizing neurons are localized in the preoptic area and adjacent sites in the rostral portion of the hypothalamus (Sherwood *et al.* 1993, Lescheid *et al.* 1997). By *in situ* hybridization, it has been shown that GnRH-II mRNA is expressed in the mid-brain, hippocampus and discrete nuclei of the hypothalamus (Urbanski *et al.* 2000). Using double-label histochemistry, recent studies demonstrated that GnRH-I and GnRH-II are expressed by two separate populations of cells in the rhesus macaque hypothalamus (Latimer *et al.* 2000). Interestingly, GnRH-II is expressed at significantly higher levels outside the brain, especially in the kidney (up to 30-fold), bone marrow (up to fourfold) and prostate (up to fourfold) in the human (White *et al.* 1998). The unique location and differential expression levels of GnRH-II within and outside the brain in a single species, including the human, suggest that it might have functions distinct from those of GnRH-I.

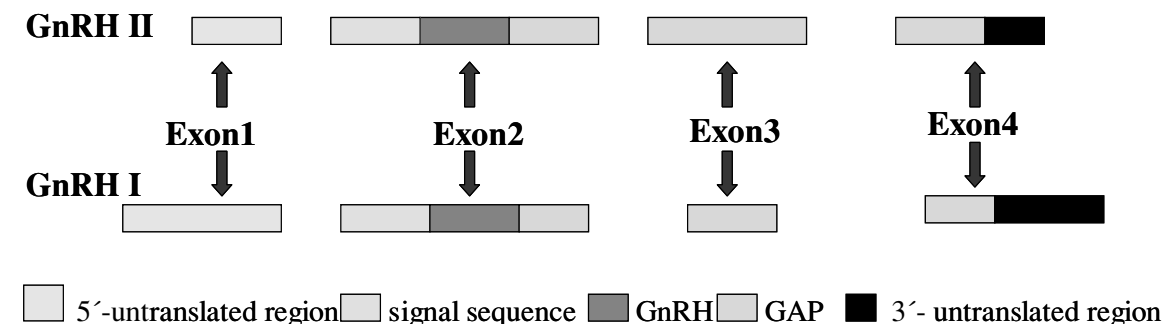


Figure 1 The structure and similarity of GnRH-I and GnRH-II genes. The GnRH genes consist of four exons and three introns and exist as part of a larger precursor gene. GAP, GnRH-associated peptide.

Cloning and molecular structure of GnRHRs

At present, two types of GnRHRs in mammals and three types in invertebrates have been characterized (Neill 2002). The type I GnRHR gene was first cloned in mice and subsequently cloned in rats, sheep, bovine and human (Kaiser *et al.* 1997), while its cDNA encodes a 327 amino acid protein in the mouse and rat. The GnRHRs expressed in human and sheep contain one more amino acid, a lysine residue in the

second extracellular loop, and encode a 328 amino acid protein. Southern blot analysis revealed that the GnRHR appears to be encoded from a single gene (Fan *et al.* 1994, Kaiser *et al.* 1997). The type I GnRHR gene consists of three exons, two introns and putative seven transmembrane (TM) domains (Fig. 2A), which are characteristics of the G-protein-coupled receptor (GPCRs) family. A highly conserved sequence, i.e. Asp-Arg-Tyr triplet, in many other GPCRs is replaced by Asp-Arg-Ser in the GnRHR. Another highly unusual feature

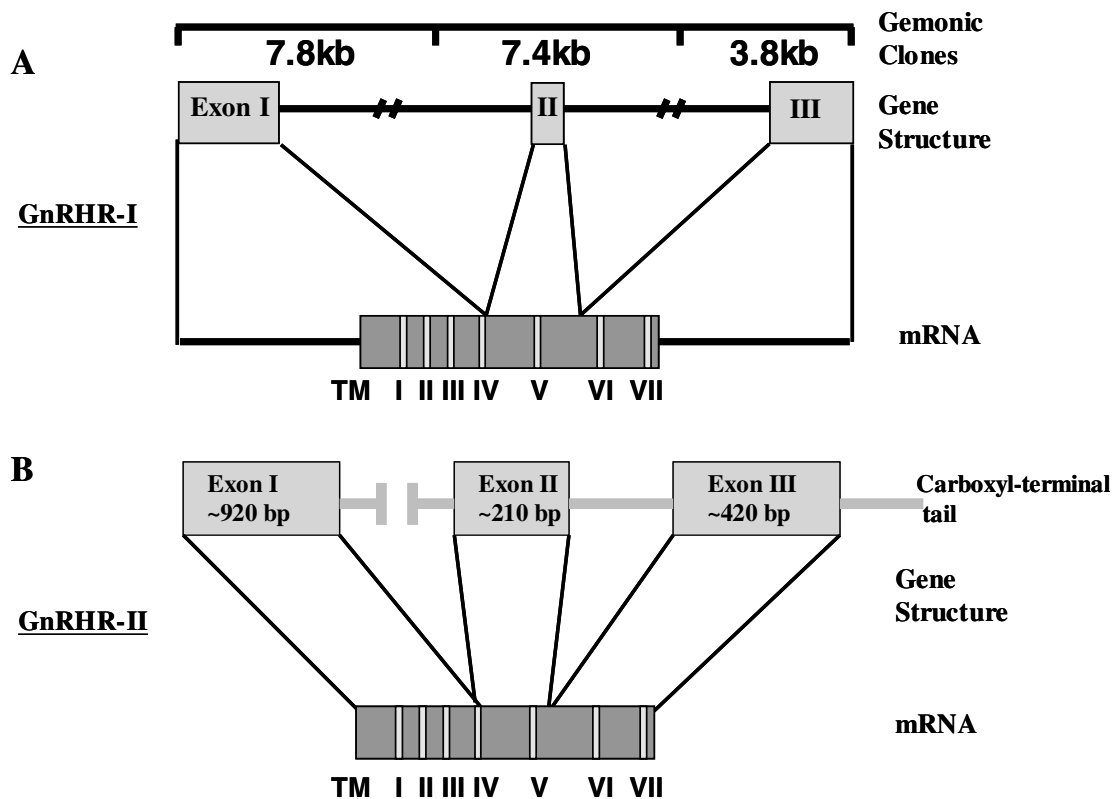


Figure 2 The structure of human type I (A) and putative type II (B) GnRHR, which consist of three exons, two introns and putative seven TM domains.

of the GnRHR is the exchange of conserved aspartate (D) and asparagine (N) residues in the second and seventh TM domain. The most unique structural feature of the mGnRHR among GPCRs is the absence of a carboxy-terminal cytoplasmic tail, a region that has been implicated in coupling to G proteins in GPCRs (Fan *et al.* 1994, Kaiser *et al.* 1997). In addition to type I GnRHR, the type II GnRHR (Fig. 2B) has been recently cloned in brain and pituitary from the goldfish (*Carassius auratus*, Illing *et al.* 1999) and mammals such as the marmoset (*Callithrix jacchus*, Millar *et al.* 2001) and the rhesus monkey (*Macaca mulatta*, Neill *et al.* 2001). This type II receptor in goldfish shares a lower identity with mGnRHR (43%) and has distinct ligand selectivity. Two subtypes of type II GnRHR are expressed in the goldfish pituitary, and each has a unique pattern of expression in the goldfish brain, ovary and liver as revealed by *in situ* hybridization studies. Unlike the type I receptor, type II GnRHR in the marmoset and rhesus monkey has a carboxy-terminal tail and resembles more closely the type II GnRHR of the fish. Only 41% (marmoset type II receptor) and 39% (rhesus monkey type II receptor) identities with the type I receptor have been reported (Millar *et al.* 2001, Neill *et al.* 2001). The mammalian type II GnRHR has been proved to be functional and specific for GnRH-II in terms of the production of inositol phosphate, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Millar *et al.* 2001, Neill *et al.* 2001). Although the type II GnRHR in human genome and various human tissues has been detected, it was not possible to find a full-length, appropriately processed transcript representing type II GnRHR in human tissues (Neill 2002). In contrast, the presence of GnRH-II receptor immunoreactivity has been demonstrated in the human pituitary and brain (Millar *et al.* 2001). Recently, three types of GnRHR (bfGnRHR-1, bfGnRHR-2 and bfGnRHR-3) have been reported to exist in a single diploid species, the bullfrog (Wang *et al.* 2001). Each GnRHR mRNA displayed a distinct spatiotemporal expression, such that bfGnRHR-1 mRNA is predominantly expressed in the pituitary, whereas bfGnRHR-2 and -3 mRNAs are expressed in the forebrain and hindbrain. When compared, the three types of bfGnRHRs have been demonstrated to have an amino acid identity of ~30% to ~35% with mGnRHRs, and ~40% to ~50% with non-mammalian GnRHRs. By functional assays, three types of GnRHR have been shown to be functional and have ligand specificity as evidenced by a ligand-dependent increase in inositol phosphate production. Salmon GnRH has been shown to have a strikingly high potency to stimulate all three receptors. In addition, cGnRH-II has been reported to have a higher potency than mGnRH in inositol phosphate production.

Distribution of ovarian GnRHs/GnRHRs

Extrapituitary action of GnRHs in the ovary has been documented, suggesting the presence of a GnRH/GnRHR system

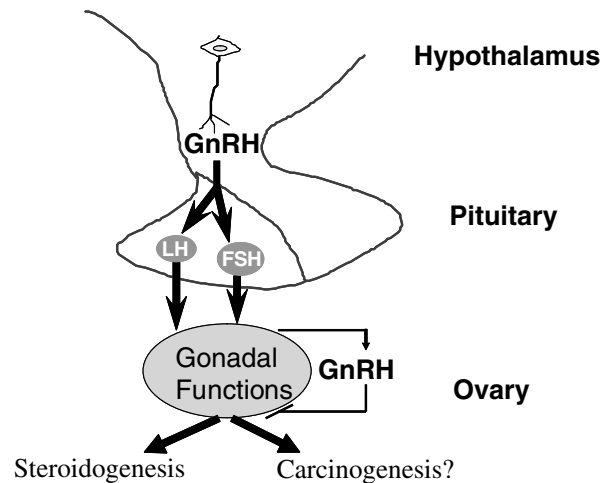


Figure 3 Extrapituitary action of GnRHs in the ovary. The presence of a GnRH/GnRHR system suggests autocrine functions in the ovary.

(Fig. 3). *In situ* hybridization studies localized GnRH-I mRNA primarily in granulosa cells of primary, secondary and tertiary follicles in the ovary (Clayton *et al.* 1992, White-law *et al.* 1995). The presence of mRNA for GnRH-I and GnRHR has also been identified in the rat ovary, human granulosa-luteal cells (hGLCs), normal ovarian surface epithelial (OSE) and ovarian cancer cells by RT-PCR amplification (Peng *et al.* 1994, Olofsson *et al.* 1995, Kang *et al.* 2000). In the testis, GnRH is present in the Sertoli cells (Bahk *et al.* 1995), whereas its receptors are expressed in Leydig cells (Clayton *et al.* 1980). Sequence analysis of the rat and human ovarian GnRHR revealed that they have a sequence identical to those found in the pituitary (Peng *et al.* 1994, Olofsson *et al.* 1995, Kang *et al.* 2000). Recently, the presence of GnRH-II in the ovarian cells such as hGLCs, normal OSE, immortalized OSE (IOSE) and primary cultures of ovarian tumour and ovarian carcinoma cell lines including CaOV-3, OVCAR-3 and SKOV-3 has been reported (Choi *et al.* 2001, Kang *et al.* 2001a).

Physiological role of ovarian GnRHs/GnRHRs

In granulosa-luteal cells and oocytes

Ovarian GnRHs have been implicated in the endocrinology of normal and malignant reproductive tissues. The recent cloning of a second form of GnRH (GnRH-II) in the primate brain has prompted a re-evaluation of the role of GnRH in reproductive functions. In the ovary, GnRH-I has been involved in a variety of both inhibitory and stimulatory responses, affecting cellular functions of ovarian cells. There is increasing evidence for a role of GnRH-I in the

regulation of atresia. During the follicular phase, GnRHR expression is high in atretic rat follicles (Whitelaw *et al.* 1995). *In vitro*, GnRH-I inhibited DNA synthesis (Saragueta *et al.* 1997) or induced apoptosis in rat granulosa cells (Billig *et al.* 1994). During the periovulatory period, GnRH-I induced transcription of several genes involved in follicular rupture and oocyte maturation, including plasminogen activator (Ny *et al.* 1987), prostaglandin endoperoxide synthase type 2 (Wong & Richards 1992) and progesterone receptor (Natraj & Richards 1993). It has been suggested that GnRH-I may be involved in the process of luteinization and luteolysis. For example, GnRH-I induced remodelling of the extracellular matrix by inducing structural luteolysis in superovulated rats through the stimulation of matrix metalloproteinase (MMP)-2 and membrane type 1 MMP in developed corpus luteum (CL), which degraded collagens type IV and type I/III respectively (Goto *et al.* 1999). During early pregnancy in the rat, GnRH-I suppressed serum progesterone levels, and increased the degree of DNA fragmentation in the CL (Sridaran *et al.* 1998, 1999). Similarly, GnRH-I induced an increase in the number of apoptotic human granulosa cells obtained during oocyte retrieval for *in vitro* fertilization (Zhao *et al.* 2000). It has been demonstrated that GnRH-I inhibited progesterone secretion in the rat and human ovary (Peng *et al.* 1994, Olofsson *et al.* 1995). However, other groups reported a stimulatory (Olsson *et al.* 1990) or no effect (Casper *et al.* 1984) of GnRH-I on progesterone production in hGLCs.

Functionally, it has been shown that GnRH-II and GnRH-II agonist (10^{-10} to 10^{-7} M) inhibited basal and human chorionic gonadotrophin (hCG)-stimulated progesterone secretion in hGLCs, which were similar to the effects of GnRH-I treatment on ovarian steroidogenesis (Kang *et al.* 2001a). Like GnRH-I, GnRH-II treatment resulted in the down-regulation of FSH receptor and LH receptor in hGLCs, suggesting that GnRH-II may exert its anti-gonadotrophic effect by down-regulating gonadotrophin receptors (Kang *et al.* 2001a). Interestingly, GnRH-II and GnRH-II agonist did not affect basal and hCG-stimulated intracellular cAMP accumulation, suggesting that the anti-gonadotrophic effect of GnRH-II may be independent of the modulation of cAMP levels (Kang *et al.* 2001a).

GnRH has been suggested to be involved in the process of fertilization. GnRH and GnRH agonists have been shown to increase the cleavage rate of bovine oocytes (Funston & Seidel 1995). Moreover, GnRH enhanced sperm-zona pellucida binding ability, which is completely blocked by co-treatment with GnRH antagonist (Morales 1998). During the luteal phase, but not the follicular phase of the menstrual cycle, both GnRH mRNA and protein have been demonstrated in the human Fallopian tube, where spermatozoa and oocytes are deposited to form zygotes (Casan *et al.* 2000).

In ovarian surface epithelium and ovarian cancer

GnRH-I and its receptor have been identified in human ovarian carcinoma, breast tumour tissues, endometrial carcinoma, as well as prostate tumours (Schally 1999), suggesting that GnRH-I may be an autocrine/paracrine regulator of tumour growth in these cancers. GnRHRs were shown to be expressed in 80% of human ovarian epithelial tumours and in numerous ovarian cancer cell lines (Emons *et al.* 1993, 1997, Miyazaki *et al.* 1997). The majority of primary ovarian cancers express GnRHRs, which may mediate direct anti-proliferative effects of GnRH analogues, suggesting a novel therapeutic approach (Volker *et al.* 2002). In normal OSE from human, a direct growth-inhibitory effect of GnRH-I was demonstrated by treatment with GnRH analogues and assessment of the proliferative index in these cells by a [3 H]thymidine incorporation assay (Kang *et al.* 2000). The anti-proliferative effect of the GnRH agonist was receptor mediated as co-treatment of normal OSE and ovarian cancer cells with antide abolished the growth-inhibitory effect of the GnRH agonist, suggesting that GnRH-I can act as an autocrine/paracrine regulator in normal OSE and ovarian cancer cells. In immortalized IOSE-29 and IOSE-29EC cells, which were generated from normal OSE cells by transfecting SV40 large T antigen and E-cadherin gene subsequently, the increasing doses of GnRH-II (10^{-9} to 10^{-7} M) induced a growth inhibition, suggesting that GnRH-II, like GnRH-I, may have an anti-proliferative effect in these IOSE cells (Choi *et al.* 2001). In ovarian (EFO-21, OVCAR-3 and SKOV-3) and endometrial cancer cell lines (Ishikawa and HEC-1A), the presence of the second type of GnRHR (GnRH-II receptor) with low affinity and high capacity has been proposed in recent studies (reviewed by Schally 1999, Grundker *et al.* 2002a) and subsequently confirmed by RT-PCR using specific primers derived from pituitary human type II GnRHR mRNA (Faurholm *et al.* 2001) and Southern blot analysis (Grundker *et al.* 2002b). In the GnRH-II and GnRH-I receptor-positive ovarian (EFO-21 and OVCAR-3) and endometrial (HEC-1A and Ishikawa) cell lines, treatment with both native GnRH-II or GnRH-I agonist triptorelin reduced cell number in a time- and dose-dependent manner, and greater anti-proliferative effect was caused by the treatment with native GnRH-II. In the GnRH-II receptor-positive but not the GnRH-I receptor-negative ovarian cancer cell line SKOV-3, only native GnRH-II but not the GnRH-I agonist was shown to inhibit cell growth (Grundker *et al.* 2002b).

Numerous *in vitro* studies have shown that GnRH and its analogues inhibit the growth of a number of GnRHR-bearing ovarian cancer cell lines. A time- and dose-dependent inhibition have been demonstrated in the growth of two ovarian cancer cell lines, EFO-21 and EFO-27, following treatments with GnRH agonist, [D -Trp 6]LH-releasing hormone (LHRH)

(Emons *et al.* 1993). In addition, treatment with GnRH agonists [D-Trp⁶]LHRH and Lupron-SR, induced a growth inhibition of the ovarian cancer cell line, OVCAR-3 (Mortel *et al.* 1986, Peterson *et al.* 1994). Interestingly, an antagonistic analogue of GnRH, SB75, also resulted in an inhibition of cell growth in OV-1063 ovarian cancer cells in a dose-dependent manner (Yano *et al.* 1994). Furthermore, the suppression of endogenous FSH and LH secretion in the pituitary gland resulted in a growth inhibition of heterotransplanted ovarian cancers following treatment with GnRH agonist in an animal model (Peterson *et al.* 1994). In a clinical trial, combined treatment with the GnRH agonist, [D-Trp⁶]LHRH, and cisplatin has been shown to improve the positive outcome as compared with patients on chemotherapy alone (Medl *et al.* 1993).

A targeted chemotherapeutic approach has been recently developed to enhance the therapeutic efficiency of GnRH analogues and reduce cytotoxicity against normal cells (Schally & Nagy 1999). The targeted cytotoxic peptide conjugates are composed of a peptide that binds to receptors in cancer cells and a cytotoxic chemical, which has a cytotoxic effect. AN-152, in which a cytotoxic chemical, doxorubicin, is linked to a peptide, [D-Lys⁶]GnRH, and AN-207 which consists of 2-pyrrolino-doxorubicin coupled to the same peptide, have been developed. These cytotoxic analogues of GnRH have been demonstrated to have a high-affinity binding for GnRHR in tumour cells, less toxic and more efficient than the use of only cytotoxic agents to reduce the growth of GnRHR-positive human ovarian, mammary or prostatic cancer cells (Szepeshazi *et al.* 1992, Kahan *et al.* 1999). In nude mice, it has been demonstrated that AN-152 is more effective and less toxic than equimolar doses of doxorubicin at inhibiting the growth of GnRHR-positive OV-1063 ovarian cancers (Miyazaki *et al.* 1997). In addition, treatment with AN-207 also resulted in an inhibition of ovarian tumour growth, OV-1063, in nude mice with less toxicity than equimolar doses of its 2-pyrrolino-doxorubicin (Miyazaki *et al.* 1999).

The exact mechanism of the GnRH-growth inhibitory effect remains to be uncovered in ovarian cancer cells. Continuous treatment with GnRH agonists, which is thought to induce the down-regulation of its receptors, resulted in an inhibition of ovarian cancer growth, and an inhibitory effect of tumour cell growth was abolished following co-treatment with a specific GnRH antagonist (Thomson *et al.* 1991, Kang *et al.* 2000), suggesting that the ovarian GnRHRs may be involved in a direct anti-proliferative effect of GnRH analogues. However, this notion is not corroborated by the observation that both antagonistic and agonistic analogues have been reported to induce growth inhibition of ovarian cancer cells (Yano *et al.* 1994, Tang *et al.* 2002). The GnRH-I antagonist, cetrorelix, induced a direct inhibition of cell proliferation in human epithelial ovarian cancer cells, and the mechanisms of the cetrorelix effect is involved in cell-cycle progression, including G1

phase cell cycle arrest coupled with down-regulation of cyclin A-Cdk2 complex levels, suggesting an up-regulation of p53 and p21 protein levels and apoptosis (Tang *et al.* 2002). Recent results suggest that the mechanism of GnRHR signalling may be mediated by phospholipase C; however, protein kinase C may not be involved in the anti-proliferative effect of GnRH in cancer cells (Emons *et al.* 1998). In addition, GnRH and GnRHR might induce an activation of down-stream phosphotyrosine phosphatase (PTP) in GnRHR-positive tumours, indicating a counteracting role against growth factors, which play a role in an activation of receptor tyrosine kinase (Lee *et al.* 1991, Imai *et al.* 1996). It has been reported that GnRH analogues counteracted the growth-stimulatory effect of epidermal growth factor (EGF) in ovarian cancer cells, suggesting that GnRH may down-regulate its receptor numbers and/or mRNA levels (Emons *et al.* 1996). Further studies have demonstrated that treatment with GnRH analogues induced a reduction of cell proliferation, through an increase in the cell portion of resting phase, G₀-G₁ (Thomson *et al.* 1991) and induction of cell death or apoptosis (Motomura 1998, Sridaran *et al.* 1998). The GnRH-induced apoptosis may be mediated by the Fas ligand-Fas system in ovarian cancer cells (Nagata & Golstein 1995). In addition, recent studies indicated that GnRH analogue may modulate a growth of ovarian cancer cells by inhibiting telomerase activity without altering the RNA component of telomerase expression (Ohta *et al.* 1998). It has been recently demonstrated that JunD activation by GnRH plays an important role as a modulator of cell proliferation and cooperates with the anti-apoptotic and anti-mitogenic actions of GnRH (Gunther *et al.* 2002).

Regulation of GnRH and its receptor in the ovary

Unlike in the hypothalamus and pituitary, the regulation of GnRH and its receptor in the ovary is poorly understood. In the rat ovary, *in situ* hybridization analysis revealed that GnRHR gene expression was dependent on the degree of follicular development and the stage of the oestrous cycle. The GnRHR expression was greatest in the granulosa cells from Graafian and atretic follicles, with lower levels of expression present in preantral, small antral follicles and CL (Bauer-Dantoin & Jameson 1995). The GnRHR mRNA levels in atretic follicles increased up to threefold on the day of pro-oestrus coincident with the preovulatory gonadotrophin surge, while the level of GnRHR gene expression in CL significantly increased between the morning of metoestrus and the afternoon of pro-oestrus (Bauer-Dantoin & Jameson 1995). Interestingly, GnRHR expression in the rat ovary is correlated with the expression of pituitary GnRHR. The highest level of receptor expression was observed in pro-oestrus, just prior to the gonadotrophin surge, and these levels were maintained throughout the gonadotrophin surge, followed by a decline in metoestrus (Bauer-Dantoin & Jameson 1995). In

preovulatory rat granulosa cells, GnRH induced an increase in the receptor levels in a dose-dependent manner, whereas LH decreased in GnRHR mRNA levels (Bauer-Dantoin & Jameson 1995). In the rat, treatment with oestradiol enhanced GnRHR gene expression in granulosa cells from growing and atretic follicles (Kogo *et al.* 1999), whereas little is known about the primate ovary. In cultured hGLCs (Peng *et al.* 1994) and OSE cells (Kang *et al.* 2000), treatment with GnRH induced a biphasic effect (up- and down-regulation) on GnRH and GnRHR mRNA expression in these cells. Interestingly, treatment of hGLCs with GnRH-II and its agonist induced a significant decrease in GnRH-II and GnRHR mRNA levels, whereas GnRH-I treatment induced a biphasic effect of GnRH-I and GnRHR mRNA, suggesting that GnRH-I and GnRH-II may differentially regulate GnRHR and its ligands (GnRH-I and GnRH-II) in hGLCs (Kang *et al.* 2001a). In addition, treatment with FSH or hCG induced an up-regulation of GnRH-II mRNA levels, while it decreased GnRH-I mRNA in hGLCs (Kang *et al.* 2001a), suggesting that GnRH-II may be under differential hormonal regulation in these cells. The treatment of hGLCs cells with hCG has been shown to inhibit GnRHR gene expression (Peng *et al.* 1994). In addition, treatment with oestrogen resulted in a dose- and time-dependent decrease in the GnRH mRNA in hGLCs. In contrast, a biphasic effect on GnRHR mRNA expression with time was observed in response to oestrogen in these cells (Nathwani *et al.* 2000). A potential interaction was demonstrated between the oestradiol/oestradiol receptor and GnRH/GnRHR systems, indicating that this cross-talk may be important in the growth regulation of normal OSE and ovarian cancer cells (Kang *et al.* 2001b). Oestrogen induced a significant down-regulation of GnRH mRNA in OVCAR-3 cells, but not in normal OSE cells. In contrast, oestrogen induced a down-regulation of GnRHR mRNA in both normal OSE and OVCAR-3 cells in a receptor-mediated manner just as tamoxifen, an oestrogen antagonist, prevented the effect of oestrogen. Using [³H]thymidine incorporation, it was demonstrated that co-treatment with oestrogen significantly attenuated the growth-inhibitory effect of a GnRH agonist in OVCAR-3, whereas no effect of oestrogen was observed in normal OSE cells (Kang *et al.* 2001b). These results suggest that the effect of oestrogen may be involved in the down-regulation of GnRH or GnRHR mRNA in normal OSE and OVCAR-3 cells.

Conclusion

In summary, ovarian GnRHs/GnRHRs have been shown to play an important role in the control of the endocrinology of reproductive tissues, affecting follicular and luteal function, ovarian cell growth and death. A variety of both inhibitory and stimulatory effects of ovarian GnRHs are summarized in Fig. 4.

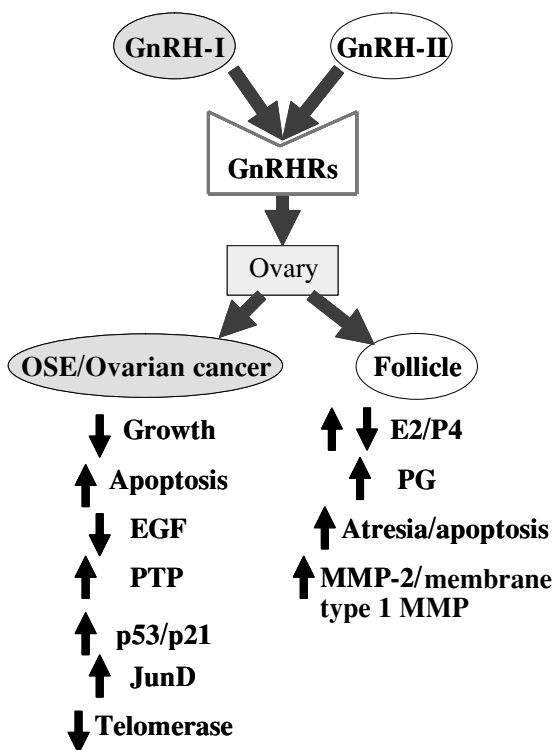


Figure 4 Actions of GnRHs in ovarian steroidogenesis and carcinogenesis. E2, oestradiol; P4, progesterone; PG, prostaglandin.

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