HUMAN GONADOTROPIN-RELEASING HORMONE-II REGULATION IN OVARIAN CANCER: MECHANISMS AND POTENTIAL FUNCTIONAL ROLE

by

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Abstract

It is increasingly apparent that GnRH-II acts as an autocrine/paracrine regulator in non-pituitary tissues, in addition to its role in the regulation of gonadotropin synthesis, and is an important player in cancer cell biology. High levels of GnRH-II and GnRHR in malignant ovarian tumors as compared with benign ovarian tissues underlies the importance of understanding GnRH-II function in ovarian cancer. In an attempt to define the regulation of *GnRH-II* in these tissues, we found that a cyclic-AMP responsive element (CRE) is critical for *GnRH-II* promoter transcription. In this scenario, the transcription factors p-CREB, C/EBPβ and CBP are recruited to this region in a temporarily-defined manner in response to cAMP/PKA signaling, thereby enhancing *GnRH-II* transcription and increasing GnRH-II mRNA levels in cancer cells of reproductive tissues. We also verified that EGF/EGFR-activated p-CREB/C/EBPβ interactions target the CRE region within *GnRH-II* promoter to enhance GnRH-II production in ovarian cancer. Importantly, EGF-stimulated *GnRH-II* expression constitutes a specific autocrine loop that contributes to ovarian cancer motility. In an attempt to define the downstream mechanisms responsible for this autocrine action of GnRH-II, we identified that MMP-2 and MT1-MMP are critical mediators of GnRH-II-enhanced ovarian cancer cell invasion. Specifically, GnRH-II acts *via* GnRHR to up-regulate 37kDa laminin receptor precusor expression which dimerizes to yield the non-integrin 67kDa laminin receptor (67LR). This leads to an increase interaction between 67LR and laminin in the extracellular matrix, and increases MMP-2 production in ovarian cancer cells. In parellel, GnRH-II/GnRHR-activated PI3K/Akt/β-catenin signaling to up-regulate MT1-MMP production which is known to be an activator for MMP-2 zymogen. Lastly, we attempted to define the implication of 67LR in high grade serous ovarian carcinoma due to its critical role in enhancing ovarian cancer progression in our *in vitro* model. However 67LR did not correlate with the overall survival of stage III & IV high-grade serous ovarian cancer patients. Overall, this study contributes to our understanding of the impact of GnRH-II/GnRHR in ovarian cancer invasive potential and provides insights into the progression of ovarian cancer and the development of new therapeutic strategies.

Preface

A version of chapter II has been published. **Poon SL**, An BS, So WK, Hammond GL, Leung PC. 2008. Temporal recruitment of transcription factors at the 3'5'-cyclic adenosine 5'-monophosphate-response element of the human GnRH-II promoter. *Endocrinology* 149:5162-71.

I was responsible for the experimental design and conducted all the experiment in this chapter. I wrote the manuscript which was revised by my supervisors. An BS and So WK have provided techniqcal assistance and advice in the initial experimental design.

A version of chapter III has been published. **Poon SL**, Hammond GT, Leung PC. 2009 Epidermal growth factor induced GnRH-II synthesis contributes to ovarian cancer cell invasion. *Mol Endocrinol* 10:1646-56.

Hammond GT was a summer student in our laboratory. He performed the Western blot for FIG. 3.3. and prepared the summary figure (FIG. 3.7). I performed all the other experiments and I wrote the manuscript which was critically revised by my supervisors.

I performed all the experiments in Chapter IV

Lau MT is a PhD candidate in our laboratory and performed the luciferase assay for FIG. 5.2. in Chapter V. I performed all the other experiments in this chapter.

Chapter VI was performed in collaboration with the scientists at the British Columbia Cancer Agency (BCCA). The immunostaining of tissue microarray was performed by Miss Christine Chow. The scoring of the staining was performed by myself, and with the help of Dr. Mark Carey. The statistical analysis was performed with help from Dr. Steve Kalloger.

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List of Abbreviations

AP-1	Activator protein 1
Akt	AKT8 virus oncogen cellular homolog
ANOVA	Analysis of variance
APC	Adenomatous polyposis coli
BRCA1	Breast cancer 1
BRCA2	Breast cancer 2
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
c-FLIP	Caspase 8 and FADD-like apoptosis regulator
ChIP	Chromatin immunoprecipitation
CRE	cAMP response element
CREB	CRE binding protein
CBP	CREB binding protein
C/EBPβ	CCAAT enhancer binding protein β
C/EBPα	CCAAT enhancer binding protein α
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
E2	Estradiol
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGR-1	Early growth response 1
ERE	Estrogen response element
ERK1/2	Extracellular signal-regulated kinase 1/2
EOC	Epithelial ovarian cancer
EMSA	Electromobility shift assay
ER	Estrogen receptor
ErbB	v-erb-b erythroblastic leukemia viral oncogene
Ets	E twenty six transcription factor family
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
Fos	FBJ murine osteosarcoma viral oncogene homolog
FSH	Follicle stimulating hormone
GAP	GnRH associated peptide
GnRH-I	Gonadotropin-releasing hormone I
GnRH-II	Gonadotropin-releasing hormone II
GnRHR	Gonadotropin-releasing hormone receptor
GPCR	G protein couple receptor
GSK3β	Glycogen synthase kinase 3 beta
hCG	Human chronic gonadotropin hormone
HGF	Hepatocyte growth factor
hGLC	Human granulosa luteinizing cells
IGF	Insulin-like growth factor
JNK	c-Jun terminal kinase
Jun	Jun oncogene
kDa	Kilodalton

KGF	Keratinocyte growth factor
LH	Luteinizing hormone
M	Micro
MAPK	Mitogen activated protein kinase
Ml	Mililiter
Min	Minute
MLK3	Mixed lineage protein kinase 3
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
MT1-MMP	Membrane type I matrix metallproteinase
MW	Molecular weight
NF-κB	Nuclear factor kappa B
OSE	Ovarian surface epithelium
P4	Progesterone
PBS	Phosphatase buffered saline
PCR	Polymerase chain reaction
PED	Phosphoprotein enriched in diabetes
PEA15	Phosphoprotein enriched in astrocyte 15
PI3K	Phosphoinositide3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PR	Progesterone receptor
RARα	Retinoic acid receptor alpha
RXRα	Retinoid X receptor alpha
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcription polymerase chain reaction
SDF-1a	Stromal cell derived-1a
SF-1	Steroidogenic factor 1
Shc	Src homology 2 domain containing transforming protein
SOS	Son of sevenless homolog
Taq	*Thermus acuaticus*, source of DNA polymerase
TGF-β	Transforming growth factor beta
TIMP	Tissue inhibitor of metalloproteinase
TM	Trans-membrane
VEGF	Vascular endothelial growth factor
Wnt	Wingless-type MMTV integration site family
37LRP	37 kDa laminin receptor precursor
67LR	67 kDa non-integrin laminin receptor
μ	micro

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CHAPTER 1. Introduction

1.1 Gonadotropin-releasing hormone (GnRH)

The hypothalamic gonadotropin-releasing hormone (GnRH, pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH2) is a decapeptide that plays a critical role in regulating mammalian reproductive development and function (1, 2). GnRH-I, the first GnRH isoform discovered in the mammalian brain, plays an important role in the regulation of the hypothalamic-pituitary-gonadal axis by stimulating the pituitary secretion of gonadotropins (luteinizing hormone and follicle stimulating hormone), which in turn regulate steroidogenesis and gamatogenesis in both male and female gonads. GnRH-II, the second isoform of GnRH, was first isolated from the chicken brain (3) and is highly conserved among vertebrates (4). Although GnRH-II (pGlu-His-Trp-Ser-His-**Gly-Trp**-Tyr-**Pro**-Gly-NH$_2$) has 70% identity with GnRH-I, it has been suggested that GnRH-II may have physiological functions distinct from those of GnRH-I due to its differential localization in the brain (5) and also its significantly higher levels of expression outside the brain (6). It has been reported that the efficiency of GnRH-II in terms of stimulating gonadotropin secretion is only around 2% that of GnRH-I (7). In the brain, GnRH-II acts as a neuromodulator (8), while the actions of GnRH-II in peripheral tissues are not entirely understood. Thus, the functional roles of GnRH-II in extra-pituitary tissues are potentially very interesting.

1.1.1 The promoter regulation of GnRH-II

Human GnRH-II is encoded by chromosome 20p13. The *GnRH-II* gene is comprised of four exons with three introns, and the prepro-GnRH-II is structurally similar to the GnRH-I precursor (6). The first exon of the *GnRH-II* gene is untranslated, whereas the second exon encodes the signal sequence, the GnRH decapeptide, the GKR processing signal and part of the

GnRH-associated peptide (GAP) residue. The third and fourth exon encode the remaining GAP residues and contain the translation termination codon and the 3' untranslated region.

The first report of *GnRH-II* regulation by Chen *et al.* demonstrated that treatment with dibutyryl cAMP significantly increases the secretion of GnRH-II and up-regulates *GnRH-II* gene expression in the TE671 cells (9). In addition, mutagenesis assays indicated that this effect of dibutyryl cAMP acts by regulating an atypical 8 bp palindromic cAMP response binding element (CRE; TGACGTCA) within the human *GnRH-II* promoter (9).

By performing deletion analysis within the human *GnRH-II* 5' flanking region using the neuroblastoma TE671 cell line, placental choriocarcinoma JEG-3 cells and ovarian carcinoma OVCAR-3 cells as models, Cheng *et al.* demonstrated that these cell lines posses similar promoter activity profiles and a minimal promoter region (-1124 to -750 bp relative to the translation start codon of the *GnRH-II* gene) is sufficient to initiate *GnRH-II* gene transcription in both the neuronal and reproductive cell types (10). Specifically, two E-box binding sites (-790 to 785 bp; -762 to 757 bp) and one Ets-like element (-779 to 776 bp) are found within the untranslated exon 1 of the human *GnRH-II* gene where they act as enhancer elements for the stimulation of *GnRH-II* transcription (10). Site-directed mutagenesis showed that these three elements act in a conjunction manner to regulate basal *GnRH-II* transcription (11). In this context, a basic helix-loop-helix transcription factor AP-4 has been found to bind to the E-box binding site within the human *GnRH-II* promoter, whereas the transcription factors that bind to the Ets-like element within the *GnRH-II* promoter remain elusive (10, 11).

In addition to enhancer elements, a repressor element has also been identified within the first intron (-641 to -636 bp; relative to the translation start codon) of the human *GnRH-II* promoter (12). Using electromobility shift assays and chromatin immunoprecipitation assays, Hoo *et al.* showed that the p65 subunit of nuclear factor kappa B (NF-κB) and the retinoic acid

receptors (RARα and RXRα) bind to this repressor element. Moreover, the over-expression of p65 in both TE671 and JEG-3 cells significantly inhibits human *GnRH-II* promoter activity and reduces endogenous *GnRH-II* expression. The over-expression of RARα and RXRα demonstrate a tissue-specific regulation of the *GnRH-II* gene, such that RARα and RXRα increase *GnRH-II* promoter activity in JEG-3 cells but have no effects in TE671 cells (12).

1.1.2 Regulation of GnRH-II in human

Human GnRH-I and GnRH-II are encoded by different genes, which are regulated distinctly in different tissues. Studies demonstrate that the *GnRH-I* and *GnRH-II* genes are each regulated by their own ligands, gonadotropins and steroid hormones (2). However, most reports only documented how individual factors affect GnRH-II mRNA levels in different tissues or cell lines but failed to examine the molecular mechanisms responsible for this effect.

Specifically, in human granulosa luteinizing cells, GnRH-I has been shown to be regulated by its own ligand in a biphasic manner (13), i.e., low concentrations of GnRH-I analog treatment (10 pM and 100 pM), results in an up-regulation of *GnRH-I* expression, whereas high concentrations of GnRH-I analog treatment (1 μM and 10 μM) represses *GnRH-I* expression. This mode of regulation has also been observed in hypothalamic neurons (14) and human ovarian epithelial cells (15). However, GnRH-II analog treatment does not have this biphasic effect. Instead, treatment with different concentrations of GnRH-II analog (10 pM and 10 μM) in human granulosa luteinizing cells results in a dramatic decrease in GnRH-II mRNA levels in these cells (13). In Ishikawa human endometrial cancer cells, GnRH-I analog treatment increases its own mRNA expression but has no effect on GnRH-II mRNA levels (16).

Gonadotropins, including follicle stimulating hormone (FSH) and luteinizing hormone (LH), differentially regulate the two *GnRH* genes *via* the activation of cAMP/PKA signal

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transduction pathways (2). This regulation results in distinct expression profiles of *GnRH-I* and *GnRH-II* in different cell systems (11). For instance, treatment of GT1-7 cells with LH or human chorionic gonadotropin (hCG) down-regulates GnRH-I mRNA levels through the involvement of cellular LH/hCG receptors (17). Using pharmacological agents to block PKA or PKC signaling pathways, the latter researchers demonstrated that PKA is the dominant signaling pathway for this repression by LH/hCG (18), thereby suggesting that transcription factors such as Fos, CREB and Jun, which are downstream molecules of activated PKA signaling, may be involved in the transcriptional inhibition of *GnRH-I* by hCG in GT1-7 cells. This observation was later verified by the same group in their extended studies which identified cis-acting elements and trans-acting transcription factors involved in the inhibition by hCG (19). However, the effect of gonadotropins on GnRH-II expression in GT1-7 cells was not examined. In human granulosa luteinizing cells, treatments with FSH or hCG decrease GnRH-I mRNA levels, but cause a significant concentration-dependent increase in GnRH-II mRNA levels (13).

Similarly, the human *GnRH-I* and *GnRH-II* genes have been found to be differentially regulated by steroid hormones. In TE-671 neuroblastoma cells, estradiol (E2) decreases human GnRH-I mRNA levels, but increases human GnRH-II mRNA levels (20). For instance, by binding to the estrogen receptor (ER), E2 acts on a putative estrogen response element within the *GnRH-II* promoter to up-regulate its expression (11). In human granulosa luteinizing cells, E2 suppresses *GnRH-I* expression, whereas GnRH-II mRNA levels increase in a concentration- and time-dependent manner after E2 treatment (21). Moreover, while progesterone (P4) regulates the expression of GnRH-I mRNA levels in TE671 cells, there is no effect of P4 on GnRH-II mRNA levels in this cell type. However, treatment of human granulosa luteinizing cells with the P4 antagonist, RU486, increases GnHR-II mRNA levels in a concentration- and time-dependent manner, while RU486 has no effect on *GnRH-I* expression (21), suggesting that endogenous P4 specifically inhibits *GnRH-II* expression in the ovary. The differential regulation

of the *GnRH-I* and *GnRH-II* genes by steroid hormones in the ovary suggests that the production of these two peptide hormones may be differentially controlled during the menstrual cycle in a temporally defined manner (11).

Other steroids, including androgens, glucocorticoids and dehydroepiandrosterone, or physiological regulators, such as insulin, melatonin, nitric oxide, retinoic acid and insulin-like growth factors, have been extensively investigated as potential regulators of the human and rodent *GnRH-I* gene. Their effects on human *GnRH-II* expression have not been studied (2, 11). Thus, it was considered important to further delineate the upstream regulators of human *GnRH-II* expression.

1.1.3 GnRH-II and GnRH receptor

The type I GnRH receptor (GnRHR) is a member of the rhodopsin-like G protein-coupled receptor (GPCR) superfamily, and it contains a characteristic seven-transmembrane (TM) domain structure (22-24). However, unlike other members of the GPCR superfamily, the human GnRHR lacks the entire C-terminal tail, which leads to its slow internalization and a lack of the rapid desensitization of the receptor (25, 26). Human GnRHR is encoded by chromosome 4 (27, 28), and the *GnRHR* gene is comprised of three exons separated by two introns (29, 30). Exon 1 encodes the N-terminal tail, the transmembrane (TM) 1, 2 and 3 domains and part of the TM 4 domain of the GnRHR protein. Exon 2 encodes the remaining portion of TM 4 and the TM 5 domain, whereas exon 3 encodes the TM 6 and 7 domains (29).

The distribution of the GnRHR in the pituitary is well documented (30-32). As for extra-pituitary sites of expression, the GnRHR has been detected in both normal human reproductive tissues and tumors derived from these tissues (2). For example, the GnRHR has been detected in endometrial cancer cell lines and in both non-malignant endometrial specimens and endometrial carcinomas (33-35), as well as in normal myometrium and uterine leiomyomas (36). In human

placental tissues, both GnRH agonist and antagonist bind specifically to the GnRHR (37). Importantly, *GnRHR* expression in placental cells (i.e., cytotrophoblast and syncytiotrophoblast cells) is altered in concert with the secretion of chorionic gonadotropin throughout pregnancy. (38-40). This variation in *GnRHR* expression may therefore be functionally important during different stages of pregnancy.

As shown by Western blotting analysis, polymerase chain reaction, and radioligand binding assays, GnRHR is detectable in prostate cancer (41, 42). In the latter report, it has been demonstrated that GnRHR is localized in the basal and luminal epithelial cells of both benign and malignant prostate tissues, but their GnRHR mRNA levels do not correlate with different histological grades (42). However, in a larger population analysis, the expression of GnRHR was found to be significantly higher in patients with demonstrated hormone-refractory prostate carcinoma than those that responded to hormonal treatment (43). GnRHR mRNA levels has also been detected in human breast cancer cell lines and in breast tumor biopsy specimens (44-46). Altogether, these studies suggest that GnRH may influence the progression of these cancers.

In the ovary, GnRHR mRNA levels show a spatial and temporal change that correlates with folliculogenesis (29). Specifically, GnRHR is not expressed in primordial, early antral or preovulatory follicles, but is detectable in luteinized granulosa cells, late follicles and developing corpora lutea (47, 48), thus suggesting that its stage-specific expression in the ovary may play a role in the regulation of ovulation, follicles atresia and luteolysis. Furthermore, GnRHR protein and mRNA have also been detected in human ovarian cancer cell lines, ovarian tumor specimens, and ovarian surface epithelium, from which ovarian carcinoma may originate (49, 50). Clinically, the level of GnRHR has been reported to be elevated in advanced stage (stages III and IV) ovarian carcinomas, and was suggested to be associated with the grading of this disease (51). In summary, these results suggest a probable correlation and functional role for increasing GnRHR levels in different tissue-derived cancers (52, 53).

Since the discovery of GnRH-II, numerous studies have sought to identify a cognate type II GnRHR. In the goldfish and monkey model, type II GnRHR has been cloned and was found to be structurally and functionally different from the type I GnRH receptor (54-56). Screening the human genome databases reveals a putative type II GnRHR gene on chromosome 1q2.2 (56, 57). Although type II GnRHR mRNA can be detected in a variety of reproductive tissues or reproductive tissue-derived cancer cells (56-60), studies have demonstrated that exon 1 contains a frameshift while exon 2 contains a premature stop codon within the open reading frame of type II GnRHR mRNA, suggesting that this gene cannot translate a conventional seven-trans-membrane receptor (61). Hence, the functionality of this human type II GnRHR is still in question.

The question of whether GnRH-II specifically acts through the type I GnRHR to exert its effects remains debatable. The ability of GnRH-II to stimulate gonadotropin secretion using monkey pituitary cells has demonstrated that the type I GnRHR is responsible for the transmission of GnRH-II signaling despite the existence of a functional type II GnRHR in this species (62). In contrast, researchers found that GnRH-II does not act through the type I GnRHR to exert its neuromodulatory action on musk shrew behavior (63). In tumor systems, Enomoto and Park (59) showed that GnRH-II acts through the type II GnRHR to exert its antiproliferative effects in DU145 prostate cancer cells. However, Limonta *et al.* reported that GnRH-II exerts its antiproliferative effects through the type I GnRHR in their study using the same DU145 prostate cancer cell line and two additional prostate cancer lines, LNCaP and PC-3 cells (64, 65). Similarly, Grundker *et al*. (66) showed that siRNA-mediated reduction of the type I GnRHR did not alter the GnRH-II-induced reduction in the proliferation of endometrial and ovarian cancer cell lines. This result suggests the existence of a functional type II GnRHR in these cells lines. In contrast, our group has demonstrated that both in endometrial cancer (67) and ovarian cancer cell lines (68-71), GnRH-II exerts antimitogenic actions through the activation of the type I

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GnRHR. Furthermore, in other cell types, Hong *et al.* verified that GnRH-II acts through the type I GnRHR to induce apoptosis in human luteinizing granulosa cells (72). These results add further support for the concept that GnRH-II exerts its effects by activating the type I GnRHR.

1.1.4 GnRH/GnRHR initiated intracellular signal transduction

GnRH achieves cell-specific signaling by activating different G proteins upon binding to the GnRHR. In extra-pituitary tissues, GnRH subtypes do not act through the $G_{\alpha q}$-11-phospholipase C signal transduction pathway that functions in the pituitary (73, 74). Instead, $G_{\alpha i}$ signaling is initiated, resulting in the activation of a variety of downstream signaling cascades including mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K), and nuclear factor kappa B (NF-κB) signaling (52, 53). Recent studies have also demonstrated that Wnt/β-catenin signaling is also involved in GnRH/GnRHR activation (75). These studies imply the initiation of several signaling cascades upon GnRHR activation (Fig. 1.1).
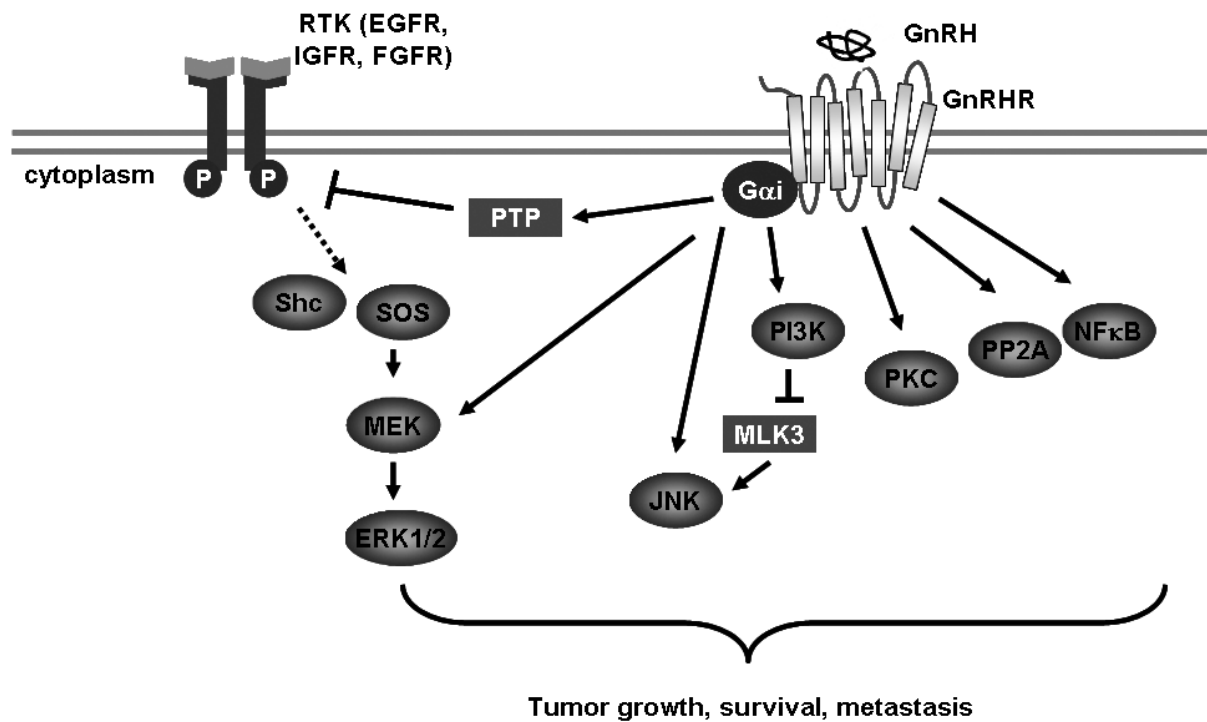
FIG. 1.1. Schematic representation of GnRHR signaling in extra-pituitary tissues. GnRH-I and GnRH-II bind with GnRHR and activate a variety of signalings to regulate tumor cell progression. GnRH, gonadotropin-releasing hormone; GnRHR, gonadoropitn-releasing hormone receptor; PI3K, phosphoinositide 3-kinase; NFκB, nuclear factor kappa light polypeptide gene enhancer in B cells; PP2A, protein phosphatise 2A; PKC, protein kinase C; MLK3, mixed lineage protein kinase 3; JNK, Jun N-terminal kinase; PTP, protein tyrosine phosphatase; RTK, tyrosine kinase receptor; EGFR, epidermal growth factor receptor; IGFR, insulin-like growth factor receptor; FGFR, fibroblast growth factor receptor; Shc, Src homology 2 domain containing transforming protein; SOS, son of sevenless homolog protein; MEK, mitogen activated protein kinase kinase; ERK1/2, extracellular signal regulated kinase 1/2.

1.1.4.1 MAPK

Several studies have shown that GnRH subtypes activate the MAPK cascade, including extracellular signal-regulated kinase (ERK), Jun N-terminal kinase (JNK), and p38 MAPK signaling in a tissue-specific and cell-specific manner. For instance, GnRH-I induces DU-145 prostate cancer cell apoptosis by activating the JNK pathway (76). This activation of JNK is achieved through the inhibition of Akt signaling and the activation of Src protein, which promotes the activity of the JNK upstream activator, MLK3 (76). In ovarian cancer cells, GnRH-I mediates cell growth by activating ERK1/2 signaling with the help of Shc and Sos proteins (77). Our laboratory has also demonstrated that GnRH-I and -II-induced MAPK signaling pathways (i.e., ERK1/2 and p38) are required to mediate cell proliferation in a PKC-dependent manner (68, 70). Recently, Cheung *et al.* demonstrated that GnRH-I acts thorough the MAPK/JNK signaling cascade to regulate ovarian cancer cell invasion (78).

1.1.4.2 PI3K

The PI3K/Akt signaling pathway has been implicated in mediating cell proliferation, survival, and invasion. Treatment with a GnRH-I agonist induces uterine leiomyoma cell apoptosis by inhibiting PI3K/Akt signaling and repressing anti-apoptotic protein expression (i.e., c-FLIP, PED/PEA15) (79). While in ovarian cancer cell line, GnRH-I and GnRH-II interfere with the activity of the PI3K/Akt signaling to regulate cell invasive properties (71). In many cases, there is a cross-talk between PI3K/Akt signaling and MAPK signaling to mediate GnRH actions. This mode of regulation has been demonstrated in prostate cancer cells in which the activation of PI3K/Akt signaling leads to the stimulation of the JNK pathway and contributes to the pro-apoptotic effect of GnRH-I (76). Similarly, in GT1-7 cells, GnRH-I can activate EGFR and the downstream PI3K/Akt signaling pathway, which can, in turn, induce ERK1/2 phosphorylation (80, 81).

1.1.4.3 Wnt/β-catenin

Recent studies have demonstrated that GPCR signaling may initiate the Wnt/β-catenin signaling cascade (Fig. 1.2). This was first reported in studies showing that prostaglandin $F_{2\alpha}$ stimulates β-catenin-dependent signaling *via* the FPB prostanoid GPCR (82-84). β-catenin may either act as an adaptor protein, which links the cadherins to the actin cytoskeleton at adherens junctions, or as a signaling molecule in the Wnt/β-catenin pathway, whereby it induces transcription of Wnt target genes *via* the T-cell factor (TCF)/lymphoid enhancer factor (LEF) transcription factor family (85-87). Without Wnt ligand stimulation, β-catenin forms a complex with adenomatous polyposis coli (APC), axin, casein kinase I and glycogen synthase kinase-3β (GSK3β). The formation of this complex regulates the phosphorylation of β-catenin and targets it for degradation in order to maintain low cellular levels of β-catenin. Stimulation of the Wnt cascade results in GSK3β phosphorylation, which releases the interaction between GSK3β and β-catenin, thereby allowing the accumulation of β-catenin in the nucleus. Consequently, mutations or alterations of any components within the β-catenin complex may lead to the dysregulation of Wnt/β-catenin target genes which affect normal physiologic homeostasis, and can promote tumorigenesis (88-91).

Recently, it has been shown that β-catenin signaling is required for the maximal induction of LHβ secretion in response to GnRH stimulation (92). This study demonstrated that GnRH induces the co-localization and physical association of β-catenin with transcription factors, EGR-1 and SF-1, on the promoter of the *LHβ* subunit to enhance gene transcription (92). Furthermore, another report demonstrated that GnRH-I and GnRH-II induce β-catenin accumulation in the nucleus, resulting in the activation of β-catenin-dependent transcription and up-regulation of Wnt/β-catenin target genes in both gonadotrope cells and GnRHR expressing

HEK293 cells (93). This study further confirmed the involvement of β-catenin signaling upon activation of GnRHR. Importantly, GnRH has been found to stimulate β-catenin signaling in a heterologous cell system (93), implying that the Wnt/β-catenin signaling cascade may be important to many peripheral tissues and cancers that express GnRHR.

1.1.4.4 Other signaling pathways

Other signaling pathways are initiated upon GnRHR activation (52, 94). For instance, a GnRH-I agonist was found to activate the nuclear translocation of NF-κB through $G_{\alpha i}$-coupled GnRHR to regulate ovarian cancer cell growth (95). The activation of this signaling pathway has also been observed in endometrial stromal cells, in which GnRH-I interferes with the tumor necrosis factor alpha-induced NF-κB signaling to inhibit the expression of interleukin-8 (96). GnRHR activation may also regulate non-RTK signaling, including proline-rich tyrosine kinase 2 (Pyk2) signaling and focal adhesion kinase (FAK). This effect was evident in human HEC-1A endometrial cancer cells in which GnRH-I and GnRH-II induced β3-integrin-dependent activation of FAK to mediate cell growth (97). In addition, GnRH-I has been shown to induce the regression of uterine leiomyoma through FAK signaling (98), and GnRH attenuates the actions of testosterone and inhibits cell growth in prostate tumors *via* the involvement of FAK signaling and the FAK cofactor, Hic-5 (99).
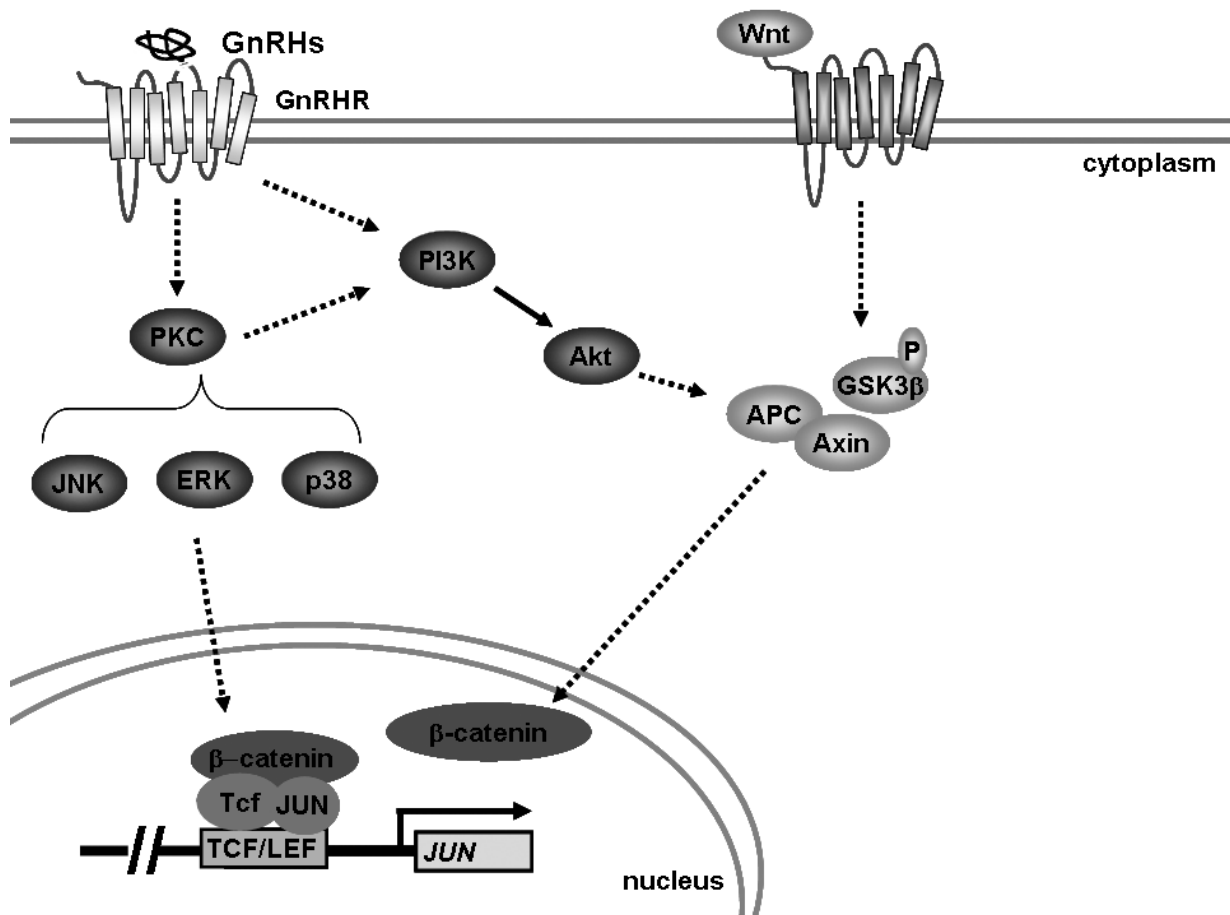
FIG. 1.2. Potential signaling pathways for permitting cross-talk between Wnt and GnRH.
GnRH/GnRHR-activated signal transduction pathway induces the translocation of β-catenin into the nucleus, thereby stimulates Wnt/β-catenin-dependent target gene transcription. GnRH, gonadotropin-releasing hormone; GnRHR, gonadotropin-releasing hormone receptor; PKC, protein kinase C; JNK, Jun N-terminal kinase; ERK, extracellar signal regulated kinase; TCF/LEF, T cell specific transcription factor/ lymphoid enhancer binding factor; JUN, jun oncogene; PI3K, phosphoinositide 3-kinase; Akt, v-akt murine thymoma viral oncogene homolog; Wnt, wingless-type MMTV integration site family; APC, adenomatous polyposis coli; GSK3β, glycogen synthase kinase 3 beta.

1.2 Ovarian cancer

Ovarian cancer is a major health problem for women and represents one of the the most lethal diseases from malignancies of the female genital tract (100). It accounts for more than 40,000 new cases each year in Europe (101) and 25,000 cases in the United States (102). Approximately 90% of ovarian cancers are epithelial (carcinomas) which can be further classified as serous (50% of ovarian cancers), endometrioid (20%), mucinous (10%), clear cell (5%), transitional, mixed, and undifferentiated, depending on their histological features (103-105).

Initially, ovarian cancers were thought to arise solely from the ovarian surface epithelium (OSE), which is a single layer of squamous to cuboidal epithelial cells that comprises the ovarian surface (103). This assumption was based on the fact that OSE possesses both epithelial and mesenchymal characteristics, so as to facilitate ovarian rupture and repair through repeated ovulatory cycles. In this context, these functions may therefore increase the susceptibility of OSE to genetic mutation and malignant transformation (106, 107). In particular, putative precursor lesions are hypothesized to originate from OSE-lined clefts and inclusion cysts in the ovarian cortex, and the prevalence of having these lesions increases with age (108-110).

On the other hand, it was noted that throughout the progression of ovarian cancer, OSE differentiates and resembles the lining of different Müllerian duct-derived regions of the female reproductive tract (97). For instance, serous, mucinous, and endometrioid ovarian carcinomas are histologically similar to the normal epithelia of the fallopian tube, endocrevix, and endometrium, respectively. This has given rise to the alternative hypothesis that epithelial ovarian cancers do not develop from metaplastic changes in the OSE but arise directly from the fallopian tube and secondary Müllerian system, including endosalpingiosis, endometriosis, and endocervicosis (111, 112). Although these different hypotheses are widely debated, recent studies have demonstrated that some tumors diagnosed as "serous ovarian adenocarcinoma" are

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more likely derived from the distal fallopian tube and then implant on the ovarian surface, particularly in women with breast cancer 1 (*BRCA1*) or breast cancer 2 (*BRCA2*) mutations (113-115). Additionally, the association between endometriosis and ovarian carcinomas of the endometrioid and clear cell subtypes is well documented (116-118).

The five-year survival rate for localized ovarian cancer is around 91-95%, compared to only 25-28% of patients that were diagnosed as advanced stage (stage III & IV) ovarian cancer (119). Many of these women with late stage ovarian cancer will exhibit relapse, or even drug-resistant disease after initial surgery and chemotherapy. Thus, the development of new treatment strategies is important in ovarian cancer cell biology. Thus far, studies of the molecular mechanisms responsible for the progression of sporadic ovarian cancer have resulted in the identification of potential target genes and their downstream molecules, and these may be targets for pharmacological inhibition or gene silencing. For example, genes that are up-regulated in cancer cells or expressed *de novo* relative to their normal counterparts may be considered as therapeutic targets. In particular, genes that are over-expressed and result in uncontrolled cell proliferation are qualified as therapeutic targets. Ideally, these target genes should not affect normal cell behavior but are required for cancer cell progression (120).

1.2.1 Growth factors regulation of ovarian epithelial cancer progression

Compelling evidence suggests that growth factors, hormones, and cytokines are important players in mediating OSE physiology (71, 121). Thus, the dysregulation within the signaling cross-talk in any of these factors, such as increased expression of receptors and their downstream elements, may contribute to the transformation and progression of epithelial ovarian carcinoma (EOC) (122, 123).

OSE expresses EGF receptors *in vivo* and in culture (124). During ovulation, the follicular fluid or blood contains high concentration of EGF which is secreted from the

luteinizing granulosa and stromal cells, thus resulting in a microenvironment that exposes the OSE cells to high levels of EGF (125, 126). Studies have suggested that EGF acts as a potent mitogen in human OSE cells and its effects may be enhanced by hydrocortisone (127). Furthermore, EGF affects the differentiation of OSE cells, in which prolonged EGF treatment leads to cell morphology changes from an epithelial to a spindle shape, with the loss of keratin, an epithelial differentiation marker (128). Further investigation demonstrated that EGF enhances cell motility and stimulates the secretion of matrix metalloproteinases, such as MMP-2 and MMP-9 (129). Taken together, EGF may constitute a periodic stimulation in OSE cells so as to induce the post-ovulatory proliferation and the epithelial-mesenchymal transition of OSE cells during follicle rupture. Failure in this mechanism will lead to the formation of epithelial inclusion cysts, which are known to be a preferential sites for malignant transformation (71).

The EGF receptor belongs to the ErbB/HER receptor tyrosine kinase family (130). It activates a variety of signaling pathways including the MAPK and PI3K cascades, which play important roles in the regulation of various cellular activities (71, 129, 131). More than 50% of ovarian tumors express the EGF receptor. Moreover, the amplification and/or over-expression of this receptor may be found in ovarian tumors compared to normal OSE. The use of antisense RNA to down-regulate the expression of this receptor results in the inhibition of cellular proliferation and attenuates the progression of tumorigenicity in human ovarian cancer cell lines, thus implicating that altered levels of this receptor play important role in this disease (132-134). Furthermore, EGF has also been shown to modulate EOC cell growth in an indirect manner. While EGF downstream signaling cross-talks to inhibit transforming growth factor-beta signaling to enhance EOC cell proliferation (135), it also interferes with the gonadoropin-mediated cell growth in these cells (69).

In addition to EGF, transforming growth factor-beta, hepatocyte growth factor, fibroblast growth factor, keratinocyte growth factor, and insulin-like growth factor have also been thoroughly investigated in terms of their physiological impact on normal OSE and EOC (71).

1.2.2 GnRH regulation of ovarian cancer progression

The expression of GnRHR has been detected in the primary cultures of ovarian carcinomas (39) and ovarian carcinoma biopsy specimens (38, 45), including both mucinous and serous subtypes (46). High expression (>80%) of GnRHR in biopsy samples (35, 44) compared with OSE supports the hypothesis that the GnRH regulatory system is involved in ovarian cancers. In line with these clinical reports, we and others have also detected the expression of GnRHR in a variety of ovarian cancer cell lines (27, 39, 41-43, 136).

The extremely short half-life of hypothalamic GnRH-I and GnRH-II suggests that their action on the ovary does not come from the systemic circulation and that there is a local source of GnRH-I and GnRH-II in ovarian cancers. Indeed, our group and others have detected GnRH-I and GnRH-II mRNA in normal OSE, immortalized OSE cells, as well as in primary cultures of ovarian tumors and ovarian cancer cell lines (43, 137, 138). In addition, the mRNA levels of GnRH-II are increased in human malignant ovarian tissues compared with benign or normal ovarian tissues (139), which points to the possibility of a GnRH-II autocrine regulation of tumor growth or motility in women with ovarian cancer (53).

GnRH-like immunoreactivity has been detected in conditioned media (49) and cell lysates (38) from ovarian cancer cell lines. While it has been reported that incubation with a GnRH-I antibody inhibits ES-2 ovarian cancer cell proliferation in a time- and concentration-dependent manner (49), others have reported a significant increase in EFO-21 and EFO-27 ovarian cancer cell proliferation after GnRH-I antiserum treatment (140). Despite this latter discrepancy, there is increasing evidence that endogenously produced bioactive GnRH acts as

an autocrine regulator in ovarian cancer cells. For instance, our laboratory has verified the existence of a GnRH-I/GnRHR autocrine loop in primary culture human OSE cells (138). In the latter studies, the GnRH-I agonist, [D-Trp6] GnRH, had a direct inhibitory effect on OSE cell growth in a time- and concentration-dependent manner, and this inhibitory effect was reversed by co-treatment with the GnRHR antagonist, antide (138). Moreover, treatment with the GnRH-I agonist stimulated the expression of GnRH-I and the GnRHR in OSE cells, further supporting the existence of a GnRH-I autocrine regulatory system in the ovary (53).

In view of the effects of GnRH-I and GnRH-II in mediating tumor cell proliferation, it has been hypothesized that GnRH subtypes may play a role in the metastatic behavior of these cancer cells. Interestingly, our group has demonstrated that GnRH-I and GnRH-II regulate the expression of several extracellular matrix-degrading enzymes in human decidual stromal cells and extravillous cytotrophoblast cells, suggesting that these peptides may be important factors during implantation (40, 68). Importantly, using ovarian cancer cell lines as cell model, others have demonstrated that GnRH-I exerts its pro-invasive effect by mediating the secretion of pro-MMP2 and pro-MMP-9 (78). Although GnRH-II demonstrates similar effects in promoting ovarian cancer cell invasion (71), the detailed mechanisms for the regulation of *GnRH-II* or its downstream signaling and its effect on the invasive potential of ovarian cancer cells remain elusive.

1.2.3 Matrix metalloproteinases in ovarian cancer progression

Interactions between the extracellular matrix (ECM) and tumor cells or the surrounding stromal cells play an important role in tumor progression (141). In particular, the secretion of matrix metalloproteinases (MMPs) is influenced by these interactions and has been shown to regulate the tumor microenvironment. In this context, increased expression and activation of MMPs has been demonstrated in a variety of cancers (142).

MMPs degrade the ECM to regulate cell behaviors relevant to cancer biology, including cancer cell growth, motility, apoptosis, differentiation, tumor angiogenesis, and immune surveillance. MMPs are divided into collagenases, gelatinases, stromelysins and matrilysins according to their specificity (143). The MMPs are also grouped according to their localization: five are secreted and three are membrane type MMPs (MT-MMPs) (144).

MMPs are synthesized as inactive zymogens (pro-MMPs), and their activation requires the proteolytic cleavage of the NH_2-prodomain (143). MMP-11, MMP-28 and MT-MMPs comprise a furin-like enzyme recognition motif between their pro-peptide and catalytic domain (145). Thus, their activation occurs intracellularly by a serine proteinase before secretion or translocation to the cellular membrane (145). The remaining MMP family members lack the furin-like domain, thereby their activation occurs extracelllary after secretion (146). Inhibitors of MMP activity include α2-macroglobulin, thrombospondin-1 and -2, and the tissue inhibitor of metalloproteinase (TIMP) family. For instance, the plasma protein, α2-macroglobulin, is the main inhibitor of MMPs in tissue fluids (146), and the inhibitory complex that is formed between α2-macroglobulin and MMPs interacts with a scavenger receptor that is responsible for its endocytotic clearance (147). In a similar manner, thrombospondin-2 forms a complex with MMP-2, which also leads to scavenger receptor-mediated endocytosis and clearance (84). On the other hand, thrombospondin-1 binds directly to pro-MMP-2 and -9 to inhibit the activation of these two MMPs (148, 149). The expression of TIMP-1, -2, -3, and -4 and their ability to inhibit MMPs are tissue specific (150). For example, studies with TIMP-2-deficient mice indicate that the proteolytic activity of MMP-2 has a direct correlation with the ratio of TIMP-2:MMP-2 in the tissues (151). Inhibitors that structurally resemble the N-terminal domains of TIMPs and either contain a C-terminal proteinase enhancer element (152) or a non-collagenase C-terminal domain (153) have been found to interfere MMPs activity. Lastly, the only known

inhibitor for membrane bound MMPs is RECK (reversion-inducing cysteine-rich protein with kazal motifs) (141).

MMP-2 and MMP-9 act in degrading the type IV collagen, a major component of the basement membrane and thus have been associated with the malignant phenotype of tumor cells (86). One of the first observations that suggested a role for MMP-9 in tumor invasion relates to the fact that the secretion of MMP-9 is associated with the metastatic phenotype of transformed rat embryo cells (154). Recently, attention has focused on membrane type 1 matrix metalloproteinase (MT1-MMP), which is an integral trans-membrane proteinase that is frequently expressed in malignant cancer cells, and has potent invasion-promoting activity (155). When expressed on the cell surface, MT1-MMP degrades the extracellular matrix (ECM) barrier adjacent to the cells to maintain the migration route (155, 156). However, other than functions as a proteinase that degrades ECM, MT1-MMP binds with cell adhesion molecules (157, 158), or latent forms of MMPs (i.e., MMP-2), and is also involved in the regulation of their respective functions (145, 159). These processing events contribute to MT1-MMP-mediated cell migration and behavior (155).

In vitro studies have demonstrated that the expression of MMP-2, -9 and MT1-MMP corresponds with the invasiveness of ovarian cancer cell lines (160-163). In fact, MMP-2, -9, and MT1-MMP are among the most studied MMPs as biomarkers for ovarian cancer. MMP-9 activity in tissue extracts increases significantly in advanced stage ovarian cancers (stage III) compared to benign tumors and has been found to be an independent prognosticator of poor survival (164). In another study of invasive epithelial ovarian cancer, high stromal expression levels of MMP-9 and MT1-MMP correlate significantly with cancer progression and act as independent prognostic markers (165). MMP-2 expression has also been correlated with ovarian cancer progression (165, 166). Elevated levels of MMP-2 in peritoneal implant cancer cells is associated with a significant risk of death in stage III ovarian carcinomas (167). Tissue MMPs

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have been shown to distinguish different histotypes of ovarian cancer, which is a significant finding given that different histotypes have different prognoses (3). A recent study showed that more than 90% of clear cell carcinomas express moderate to high levels of MMP-2 or MT1-MMP, compared with 30% to 55% of the other ovarian cancer histotypes (serous, endometrioid, and mucinous), whereas MMP-9 is expressed more widely in other histotypes (168). Importantly, the cellular source of MMPs must be considered when evaluating MMPs as ovarian cancer biomarkers. For example, high MMP-9 levels in the cancer cells themselves are associated with longer survival, whereas high stromal MMP-9 levels are associated with shorter survival, suggesting a dual role for MMP-9 during ovarian cancer progression (169). The results of these studies lead to interest in the potential use of MMP inhibitors to treat ovarian cancer. An *in vivo* model in which xenograft mice were administered intraperitoneally with synthetic MMP inhibitors demonstrated that these treatments lead to an increase survival rate in the mice implanted with human ovarian carcinoma (170). Currently, using MMP inhibitors, such as Batimastat and Marimastat, have been shown to be successful in pre-clinical studies. Nevertheless, clinical trials have revealed complications such as local pain and irritation which may result from low drug specificity (171). To address this problem, researchers are also developing therapies that specifically target individual MMP by performing gene knock-down. These *in vitro* studies have shown a promising reduction in the invasive potential of ovarian cancer cells (172).

1.3 67 kDa non-integrin laminin receptor (67LR)

The 67 kDa non-integrin laminin receptor (67LR) was first extracted from the murine melanoma cell membranes and purified by laminin-sepharose affinity chromatography (173). It was later found in human breast carcinoma cells (174) and normal muscle cells (175). The 67LR exhibits a high affinity (dissociation constant (Kd) of 2 nM) and specificity for laminin (173,

176). An approximately 1.7 Kb mRNA for 67LR was identified by Northern blot analysis, and the 888 nucleotide coding sequence for 67LR is translated into 295 amino acids resulting in a protein with a molecular mass of approximately 32.8 kDa. Western blotting demonstrated that this precursor protein can be detected in a variety of cell lysates with a molecular mass of 37 kDa. It was eventually named the 37 kDa laminin receptor precursor (37LRP) (177). Although studies have suggested that 37LRP dimerizes to form the mature 67LR (178, 179), the dimerization mechanism remains elusive.

The 37LRP is highly conserved through evolution in both vertebrates and invertebrates, suggesting that this protein may possess multiple functions in different tissues (180, 181). For example, the cDNA of 37LRP also encodes the ribosomal protein p40, suggesting a role in translation (181). In addition, studies have demonstrated that 37LRP is involved in the life cycle of prions and acts as a receptor for cellular prions (182, 183). Other studies have reported that 37LRP is identical to the oncofetal antigen protein expressed in tumors (184).

1.3.1 The functional role of 67LR in normal cells

1.3.1.1 Interactions with laminin

It is well known that 67LR interacts directly with laminin *via* two regions within the C-terminal domain: a carboxy-terminal region (205-229) that binds YIGSR on the β1 chain of laminin (185, 186), and a region encompassing amino acids 161-180, known as the peptide G domain (187-189).

Using a protease-resistant laminin fragment, a laminin-like EGF domain within the laminin-I β1 short arm (YIGSR) was identified as the minimal sequence required for binding with 67LR (190-192). The corresponding sequence that binds with this YIGSR domain has been mapped to the α-helical peptide residues of human 67LR (205-229) (185, 193). A second laminin interaction site which binds to laminin with a higher affinity (Kd =50 nM) was also

discovered (187). This domain spans residues 161-180 of 67LR (187, 194) and is known as the peptide G domain (IPCNNKGAHSVGLMWWMLAR). Studies have shown that both the 205-229 and the 161-180 regions of 67LR may bind with the YIGSR region within the laminin-1 β1 chain. (195). In fact, the peptide G domain also interacts with the sulfated polysaccharide heparin. Thus, heparin competes with laminin in binding with peptide G, resulting in the differential regulation of the interaction between 67LR and the basement membrane (196, 197). Deletion of the palindromic sequence LMWWML within the peptide G domain results in the instability of 67LR and a lower affinity of 67LR for laminin, suggesting that this palindromic sequence plays an important role in the interaction of 67LR with laminin. Overall, the interaction sites of 67LR with laminin are different from the sites recognized by integrins (180, 185, 188, 198), thus allowing a higher overall binding affinity and also a broader range of binding and signaling options.

1.3.1.2 67LR as a co-receptor

In addition to its direct binding with laminin, 67LR also facilitates the interaction between laminin and integrins. This notion was supported by reports demonstrating that the laminin-induced membrane localization of α6 and β4 integrin subunits is accompanied with increased levels of cell surface 67LR in human carcinoma cells (199, 200). Ultimately, the treatment of cancer cells with cytokines resulting in the reduction of α6 integrin subunit expression also significantly decreases the amount of cell surface 67LR (200). In addition, siRNA-mediated depletion of the α6 integrin subunit results in a proportionate reduction in 67LR cell membrane localization, despite the fact that there is no change in the total amount of mature 67LR or the total level of 37LRP. This result indicates that the siRNA treatment does not affect the overall expression of 37LRP (200). By contrast, the amount of cell surface 67LR increases in response to up-regulated α6 integrin, suggesting that these two molecules interact

with each other and may be co-regulated in terms of their localization. Indeed, an α6 monoclonal antibody also pulled down 67LR during co-immunoprecipitation, indicating that the interaction of 67LR and α6 integrin might initially occur in the cytoplasm and then may eventually co-translocate to the cell surface (200). The close association of these two receptors suggests that they may be co-regulated in a variety of processes during interaction with laminin.

1.3.2 The functional role of 67LR in tumor invasion and aggressiveness

Laminin is one of the major adhesion substrates for invasive cancer cells. Cells interact with laminin *via* several cell surface laminin-binding proteins, including integrins α3β1, α6β1, α6β4 and α7β1, dystroglycan, as well as 67LR (201). Studies have demonstrated that tumor cells with higher levels of cell surface laminin receptors not only show increased interaction with laminin but also with the vascular basement membrane (174), thereby enhancing metastatic potential (173). Thus, researchers have been trying to delineate the role of 67LR in cancer progression and metastasis. For instance, increased 67LR expression has been found in a variety of carcinomas and is positively correlated with the aggressiveness or metastatic potential of these cancers (177). In line with these clinical reports, *in vitro* studies have demonstrated that antiserum against 67LR has an inhibitory effect on cell adhesion (178). In addition, shRNA-mediated down-regulation of 67LR reduces the metastatic spread of human melanoma in nude mice (189). Interestingly, it was demonstrated that the laminin-binding peptide G domain within the 67LR plays an important role in increasing the affinity of the cells for laminin and can also increase the number and affinity of α6-containing integrins, thereby contributing to cancer cell progression (188, 202). Treatment of a synthetic peptide G with laminin-1 led to a conformational change in laminin and the subsequent remodeling of the tumor microenvironment and invasiveness *in vivo* (203, 204). Furthermore, this peptide G remodeling

selectively increases the expression of MMP-2, indicating its potential functional role in modulating tumor cell invasive properties (202).

Laminin promotes both the proliferation and migration of ovarian cancer cells (96). Clinical studies showed that 67LR levels correlate with the presence of malignancy (205), high histological grade (206), suboptimal debulking (205) and poor outcome (205, 206). Nevertheless, the sample sizes of the above reports were small. In fact, a controversial outcome was reported. In this later study, researchers focused on patients who were diagnosed with serous ovarian carcinoma, and according to their immunohistochemistry results, the levels of 67LR in both effusions and solid tumors were of marginal significance as a diagnostic marker (207). Hence, further study will be required to delineate the role of 67LR in ovarian carcinoma.

1.3.3 GnRH and 67LR

GnRH-I and GnRH-II induced the production of 67LR in both normal and leukemic T cells (20). In this study, Chen *et al.* demonstrated that T cells induce GnRH-I and GnRH-II and treatment of these peptide hormones in normal or cancerous human or mouse T cells increases the cell surface expression of 67LR. In addition, this regulation of 67LR by GnRH-I and GnRH-II resulted in increasing cell adhesion to laminin, enhanced cell chemotaxis toward the cytokine SDF-1a, and augmented the metastasis of T lymphoma into the bone marrow and spleen *in vivo*. Thus, the authors of this study suggested that GnRH-II may play a role in immune system and T cells in particular. Furthermore, they proposed that interactions of GnRH-I or GnRH-II with normal T cells may be beneficial, while the effects of these peptide hormones on malignant T cells may be detrimental. Thus, suggesting that these potential differences in normal and cancerous may have important clinical implications (20).

1.4 Hypothesis and objectives:

Gonadotropin-releasing hormone (GnRH) is the central neuroendocrine regulator of the hypothalamic-pituitary-gonadal axis. Multiple structural variants of GnRH are present in vertebrates. GnRH-II, the second isoform of GnRH, was first isolated from the chicken brain and is highly conserved among vertebrates. Studies have suggested that it is unlikely to be a primary regulator of gonadotropin release. Instead, it has been suggested that GnRH-II may have physiological functions distinct from those of GnRH-I due to its differential localization in the brain and also its significantly higher levels of expression outside the brain. Our laboratory and others have shown that GnRH-II and its receptor, GnRHR, are expressed in normal ovaries and in ovarian carcinomas. Furthermore, exogenous treatment of GnRH-II enhances the invasiveness of ovarian cancer cells, suggesting that GnRH-II/GnRHR may play an important functional role in the progression of ovarian carcinoma. Thus, my overall hypothesis for this project is that GnRH-II/GnRHR may act as an autocrine/paracrine regulator in the physiological setting of ovarian cancer.

The specific objectives of this work are:

Objective 1. To define the transcriptional regulation of human *GnRH-II* promoter in extra-hypothalamus tissues. (Chapter II)

(I) The transcriptional mechanism involving the cyclic AMP responsive element (CRE) within *GnRH-II* promoter was determined.

(II) The effects of individual transcription factors (i.e. p-CREB, C/EBPβ, CBP) on GnRH-II mRNA levels defined from (I) were determined.

Objective 2. To identify the upstream growth factors involved in the regulation of human *GnRH-II* promoter activity. (Chapter III)

(I) The effects of EGF on human *GnRH-II* promoter activity and GnRH-II mRNA level in ovarian cancer cells were determined.

(II) The regulation of EGF on the CRE within *GnRH-II* promoter was determined.

(III) The role of EGF-induced GnRH-II production in ovarian cancer cell invasiveness was determined.

Objective 3. To delineate the functional role of GnRH-II/GnRHR system in ovarian cancer (Chapter IV & V)

(I) The role of GnRH-II/GnRHR as an autocrine regulator in ovarian cancer cell invasiveness was tested. (Chapter IV)

(II) Downstream molecules (i.e. 67LR, MMP-2, MT1-MMP) involved in GnRH-II/GnRHR autocrine regulation were defined. (Chapter IV)

(III) The role of 67LR in ovarian cancer cell invasiveness was investigated. (Chapter IV)

(IV) The downstream signaling molecules (i.e., PI3K/Akt, β-catenin) involved in GnRH-II-enhanced ovarian cancer cell invasiveness were determined. (Chapter V)

(V) The downstream molecule (i.e., MT1-MMP) that responds to GnRH-II-activated signal transduction pathways was defined. (Chapter V)

Objective 4. To determine the implication of downstream mechanism activated by GnRH-II in ovarian cancer patient specimen. (Chapter VI)

(I) I examined the relationship between 67LR and the overall survival or prognosis in high-grade serous ovarian carcinoma (stage III & IV) using tissue microarray output.

CHAPTER 2. Temporal recruitment of transcription factors at the cAMP-responsive element of the human *GnRH-II* promoter[1]

2.1 Introduction

In humans, the two gonadotropin-releasing hormone (GnRH-I and GnRH-II) genes share the same structural organization but are regulated by unique regulatory elements within their promoter sequences (2), as well as in other regions of the human *GnRH-II* gene (10, 12). We have previously identified a minimal promoter region that includes two enhancer elements (E-boxes)/ and an ETS-like element in the un-translated exon 1 of the human *GnRH-II* gene (10), and others have found an atypical (agacgtca) cAMP-response element (CRE), positioned at nucleotide sequence -860 to -853 bp relative to the translation initiation codon (10) in the *GnRH-II* promoter, which responds to dibutryl- cAMP in human TE671 neuroblastoma cells (9).

Activation of the cAMP response element binding protein (CREB) *via* activation of PKA is a prerequisite for CRE-mediated alterations in gene expression (208-210). Through its highly conserved structure (211), unphosphorylated CREB can dimerize and bind DNA, but phosphorylation of CREB at serine 133 appears to increase its affinity for some promoter sequences, and notably those with atypical CREs (212). The major effect of CREB phosphorylation at this site is the recruitment of transcriptional co-activators such as the CREB binding protein (CBP) (213), which augment cAMP-induced transcription (214, 215). Other domains of CREB can also recruit other nuclear proteins to modify its transcriptional activity; for example, CCAAT/enhancer binding protein family members (215, 216) and SF-1 (196, 217) modulate CREB transcriptional activity in different ways (213).

The present study set out to define the transcriptional machinery targeting the CRE of the *GnRH-II* promoter in JEG-3 and OVCAR-3 cells. These cell lines co-express GnRH-I and

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GnRH-II and are valuable models for examining transcriptional regulation of the human *GnRH-II* gene. We demonstrate that stimulation of JEG-3 or OVCAR-3 cells with 8-bromo cAMP increases CREB phosphorylation at serine 133 within 2 h, and induces interactions between phosphorylated CREB (p-CREB), CBP and C/EBPβ in a temporally-defined manner consistent with the timing of their assembly at the CRE within the human *GnRH-II* promoter over 1-4 h. These data provide insight into the molecular mechanisms through which the classical cAMP-PKA signaling cascade activates human *GnRH-II* gene transcription in cancer cell lines of reproductive tissue origin.

2.2 Materials and methods

Cells and cell culture

Human TE671 neuronal medullablastoma cells, human OVCAR-3 ovarian adenocarcinoma cells, and human JEG-3 choriocarcinoma cells were obtained from Ameriacan Type Culture Collection (Manassas, VA, USA). The gonadotrope-derived αT3 cell line was provided by Dr. P.L. Melon (Department of reproductive medicine, University of California, San Diego, CA, USA). The cells were maintained in DMEM (Invitrogen Inc., Burlington, ON, Canada) supplemented with 10% fetal bovine serum (FBS; Hyclone Laborataries Inc., Logan, UT, USA). Cultures were maintained at 37 C in a humidified atmosphere of 5% CO_2 in air. The cells were sub-cultured when they reached about 90% confluence using a trypsin/EDTA solution (0.05% trypsin, 0.5 mM EDTA).

Plasmid construction and reporter gene assays

A full-length human *GnRH-II* promoter construct (pGL2-2103 ∕ +1-Luc) was generated by PCR amplification from human genomic DNA using sequence-specific primers followed by

subsequent cloning into the promoter-less pGL2-Basic vector (Promega, Madison, WI, USA). Transient transfections were carried out using Lipofectamin 2000 Reagent (Invitrogen) following the manufacturer's suggested procedures. To correct for transfection efficiencies, the Rous sarcoma virus (RSV)-*lacZ* plasmid was co-transfected into the cells with the *GnRH-II* promoter-luciferase construct. Briefly, 5×10^5 cells were seeded into six-well tissue culture plates the day before transfection. One microgram *GnRH-II* promoter-luciferase construct and 0.5 μg RSV-*lacZ* plasmid were co-transfected into cells grown in standard culture medium containing FBS. In some experiments, 150 nM siCREB, 150 nM siCBP and 150 nM siC/EBPβ or their control siRNA oligonucleotides (Qiagen Inc., Mississauga, ON, Canada) were co-transfected with the reporter plasmids. After 6 h, 2 ml of serum-free medium was added and the cells were further incubated overnight (18 h). The culture medium was then removed and the cells were treated with 8-bromo cAMP in serum free medium for the times indicated. Cellular lysates were collected with 150 μl reporter lysis buffer (Promega) and assayed for luciferase activity. The β-Galactosidase Enzyme Assay System (Promega) was used to measure β-galactosidase expression from the (RSV)-*lacZ* plasmid, and promoter activities were expressed as luciferase activity/β-galactosidase activity.

Reverse transcription quantitative real-time PCR (RT-qPCR)

After treatment with 8-bromo cAMP, medium was removed from the culture dish and RNA was extracted using Trizol (Invitrogen). The RNA concentration was measured based on the absorbance at 260 nm, and its integrity was confirmed by agarose-formaldehyde gel electrophoresis. Total RNA (2.5 μg) was reverse-transcribed into first-strand cDNA (GE Healthcare Bio-Science, Piscataway NJ, USA) following the manufacturer's procedure. The primers used for RT-qPCR were designed using the Primer Express Software v2.0 (Applied

Biosystems, Foster City, CA, USA). The primers for GnRH-II mRNA are: sense, 5'-CTGCTGACTGCCCACCTT; and antisense, 5'-GCTTTCCTCCAGGGTACC AG. The reactions were set up with 16.5 μl SYBR Green PCR Master Mix (Applied Biosystems). All RT-qPCR experiments were run in triplicate and a mean value was used for the determination of mRNA levels. Negative controls containing water instead of sample cDNA were used in each experiment. Relative quantification of the mRNA levels of GnRH-II was performed using the comparative Cq method with GAPDH as the reference gene and with the formula $2^{-\Delta\Delta Cq}$.

Nuclear protein extraction and immuno-precipitation

Briefly, cells were washed with cold PBS and harvested with 1 ml solution A (10 mM Hepes, pH 7.9, 10 mM KCl, 10 mM EDTA, 0.5 mM dithiothreitol, 1 μg/ml aprotinin, and 1 μg/ml protein inhibitor cocktail). Cell lysates were transferred to 1.5 ml centrifuge tubes and placed in an orbital rocker for 10 min at 4 C. Nuclear pellets were obtained by centrifugation at 14,000 g at 4 C for 10 min, and supernatants were collected for cytoplasmic protein. Nuclear pellets were re-suspended in solution B (100 mM HEPES, pH 7.9, 2 M NaCl, 5 mM EDTA, 50% Glycerol) and placed in an orbital rocker for 2 h at 4 C. After centrifugation at 14,000 g at 4 C for 5 min, supernatants containing the nuclear protein extracts were removed and stored at -80 C.

Immuno-precipitation was conducted according to the manufacturer's recommended protocol (Upstate, MA, USA). Briefly, nuclear extracts were incubated with p-CREB antibody (10 ug/ml), CBP antibody (10 ug/ml) and C/EBPβ antibody (10 ug/ml) individually followed by the antibody capture affinity ligand provided by the immuno-precipitation kit at 4 C overnight. The immuno-precipitated proteins were then subjected to electrophoresis on an 8% SDS-PAGE gel and detected with appropriate antibodies.

Chromatin immuno-precipitation (ChIP)

All reagents, buffers and supplies were included in a ChIP-IT^{TM} kit (Active Motif, Inc., Carlsbad, CA). Briefly, the cells were cross-linked with 1% formaldehyde for 10 min at room temperature. After washing and treatment with glycine Stop-Fix solution, the cells were re-suspended in lysis buffer and incubated for 30 min on ice. The cells were homogenized and nuclei were re-suspended in shearing buffer, and subjected to pre-optimized ultrasonic disruption conditions to yield 100-500 bp DNA fragments. The chromatin was pre-cleared with Protein G beads and incubated (overnight at 4 C) with 1 µg of the following antibodies: negative control mouse IgG (Active Motif), p-CREB antibody (Cell Signaling Technology Inc., Danvers, MA, USA), CBP antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), C/EBPβ antibody (Santa Cruz). Protein G beads were then added to the antibody/chromatin incubation mixtures and incubate for 1.5 h at 4 C. After extensive washing, immuno-precipitated DNA/protein complex was removed from the beads by elution buffer. To reverse cross-links and remove RNA, 5 M NaCl and RNase were added to the samples and incubated at 65 C for 4 h. The samples were then treated with proteinase K for 2 h at 42 C and the DNA was purified using gel exclusion columns. The purified DNA was subjected to PCR amplification (1 cycle of 94 C for 3 min; 40 cycles of 94 C for 20 sec; 64 C for 30 sec and 72 C for 30 sec) for the CRE site (-860/-853 bp) within the *GnRH-II* promoter using specific forward (5'-CCAGCCTAAAGCAAGAGTCC) and reverse (5'- GTCTATAAATCCTGGGGC CA) primers. As an input control, 10% of each chromatin preparation was used. The PCR products (213 bp) were resolved by electrophoresis in a 2.5% agarose gel and visualized by ethidium bromide staining. The ChIP assay was performed at least three times, and consistent data were obtained between experiments.

Data analysis

Reporter gene assays and real time PCR data are shown as the mean ± SEM of three independent experiments. Data were analyzed by one-way ANOVA, followed by test using the computer software PRISM (GraphPad Software Inc., San Diego, CA, USA). Data were considered significantly different from each other at $p < 0.05$.

2.3 Results

Human GnRH-II promoter activity is enhanced by 8-bromo cAMP in αT-3, TE671, OVCAR-3 and JEG-3 cells

When αT-3 (Fig. 2.1A), TE671 (Fig. 2.1B), OVCAR-3 (Fig. 2.1C) and JEG-3 (Fig. 2.1D) cells were transfected with a *GnRH-II* promoter luciferase reporter gene and treated with 1 mM 8-bromo cAMP for 8 h and 24 h, robust luciferase activity was determined in all cell lines. This indicates that cAMP may be a common second messenger involved in the up-regulation of *GnRH-II* transcription in these different cell types.

Human GnRH-II promoter activity and mRNA levels are enhanced by 8-bromo cAMP in a time-dependent manner

It is known that GnRH-II has an anti-proliferative effect on human ovarian surface epithelial cancer cells (50, 69, 70), as well as a potential to alter the invasiveness of human placental tissues (68). Thus, we chose human JEG-3 and OVCAR-3 cells as *in vitro* models to further study the molecular controls of *GnRH-II* promoter activity in response to cAMP. We first examined the activation of the *GnRH-II* promoter in JEG-3 and OVCAR-3 cells by transfecting them with a *GnRH-II* promoter-luciferase reporter gene construct and then stimulating them with 1 mM 8-bromo cAMP for increasing times. Fig 2.2 shows that 8-bromo

cAMP activates the *GnRH-II* promoter in a time-dependent manner, and that this reached a maximum at 24 h in JEG-3 cells (Fig. 2.2A) and at 36 h in OVCAR-3 cells (Fig. 2.2B). A cAMP dose response was performed and 1mM cAMP was found to elicit a maximal response in both JEG-3 (Fig. 2.2C) and OVCAR-3 (Fig.2.2D) cells. In parallel experiments, total RNA preparations of untreated or 8-bromo cAMP-treated JEG-3 and OVCAR-3 cells for different periods of time were subjected to real-time RT-PCR measurements of GnRH-II mRNA levels. The results showed that 1 mM 8-bromo cAMP enhanced GnRH-II mRNA levels in a time-dependent manner (Fig. 2.2E and F). In addition, the PKA inhibitor, H89, attenuated the 8-bromo cAMP and forskolin-induced *GnRH-II* promoter activity (Fig. 2.2G and H), suggesting that the *GnRH-II* promoter activation involves the classical cAMP/PKA signaling pathway.

8-bromo cAMP increases CREB phosphorylation in JEG-3 and OVCAR-3 cells

To further verify the involvement of CREB phosphorylation in *GnRH-II* activation, JEG-3 and OVCAR-3 cells were treated with 1 mM 8-bromo cAMP for 2, 4, 8, 16 and 24 h. Western blot analysis showed that 8-bromo cAMP up-regulated the phosphorylation of CREB in a time-dependent manner (Fig. 2.3). Analysis of the p-CREB to CREB ratio indicated that treatment of the cells with 8-bromo cAMP significantly increased p-CREB levels by 3 fold within 2 h, and that this was sustained over the 8 - 16 h treatment time in both JEG-3 (Fig. 2.3A) and OVCAR-3 cells (Fig. 2.3B).

CBP and C/EBPβ interact specifically and in a temporally-defined manner with p-CREB in JEG-3 and OVCAR-3 cells after 8-bromo cAMP stimulation

Immuno-precipitation experiments were conducted to determine the interaction of p-CREB with its potential co-activators including CBP, C/EBPα, C/EBPβ and SF-1.

Administration of 1 mM 8-bromo cAMP to both JEG-3 and OVCAR-3 cells enhanced the association of CBP and C/EBPβ with p-CREB in a time-dependent manner, while there was no effect on C/EBPα and SF-1 immuno-precipitations (Fig. 2.4A and B). More importantly, the interaction between p-CREB and CBP increased progressively from 4 - 16 h after cAMP stimulation, while C/EBPβ associates with p-CREB earlier (i.e., within 2 h) and is sustained to 8 h in JEG-3 cells (Fig. 2.4A) and 16 h in OVCAR-3 cells (Fig. 2.4B). Furthermore, reciprocal immuno-precipitations confirmed the interaction of p-CREB with CBP and C/EBPβ (Fig. 2.4C and D).

CREB, C/EBPβ and CBP are all required to mediate changes in GnRH-II expression in response to 8-bromo cAMP stimulation

Specific siRNA oligonucleotides were used to knock down endogenous CREB, C/EBPβ and CBP protein levels to verify their involvement in *GnRH-II* expression in OVCAR-3 and JEG-3 cells. In these experiments, 8-bromo cAMP significantly induced *GnRH-II* promoter activity (24 h) and GnRH-II mRNA levels (16 h) in cells co-transfected with control siRNA, while co-transfection with CREB, CBP or C/EBPβ-specific siRNAs compromised these effects of 8-bromo cAMP (Figs. 2.5A-C, and 2.6A-C).

8-bromo cAMP increases p-CREB, CBP and C/EBPβ association with the GnRH-II promoter in a temporally-defined manner

To determine how 8-bromo cAMP treatment of JEG-3 and OVCAR-3 cells influences p-CREB, CBP and C/EBPβ interactions with the CRE of the endogenous *GnRH-II* promoter, chromatin immuno-precipitation assays (ChIPs) were performed. Cross-linked, sheared chromatin from 1, 2 and 4 h 8-bromo cAMP-treated JEG-3 and OVCAR-3 cells were immuno-

precipitated with p-CREB, CBP and C/EBPβ antibodies respectively, and the recovered DNA was subjected to PCR using primers specific to the CRE region (-860/-853) of the *GnRH-II* promoter. As shown in figure 7, a 213 bp PCR product was amplified from p-CREB-, CBP- and C/EBPβ-immuno- precipitated DNA samples in both JEG-3 (Fig. 2.7A) and OVCAR-3 (Fig. 2.7B) cells. In contrast, little or no PCR product was observed with DNA recovered when control IgG was used for the immuno-precipitation. These data reveal the specific associations between p-CREB, CBP and C/EBPβ at the CRE region of the *GnRH-II* promoter in these cancer cells after their treatment with 8-bromo cAMP. Furthermore, in the absence of 8-bromo cAMP-treatment, ChIP analysis using p-CREB, CBP or C/EBPβ antibodies indicated no association of these factors with the *GnRH-II* CRE region. However, a PCR product was observed after 1 h with the p-CREB ChIP and the apparent abundance of this increased at 2 - 4 h of cAMP stimulation, whereas no C/EBPβ or CBP or immuno-precipitated products were observed at 1 h in both JEG-3 and OVCAR-3 cells (Fig. 2.7A and B). At 2 h after cAMP stimulation, PCR products were first observed with both the C/EBPβ and CBP ChIPs. Although the C/EBPβ ChIP PCR product remained constant between 2 and 4 h, the CBP ChIP PCR product increased further in abundance at 4 h in both cell lines (Fig. 2.7A and B). Overall, these data suggest that 8-bromo cAMP promotes p-CREB, C/EBPβ and CBP associations with the *GnRH-II* CRE in a temporally-defined manner.

2.4 Discussion

In extra-pituitary tissues, such as the placenta, ovary and endometrium, GnRH-II mimics and exceeds the activities of GnRH-I (2), and this is manifest in different biological responses. For instance, the anti-proliferative effects of GnRH-II in ovarian cancer cells are more potent than those of GnRH-I (58), and GnRH-II is also a more effective regulator of leptin and hCG

secretion in human placenta (218, 219). Immunoreactive GnRH-II is abundant in normal ovarian surface epithelial cells, immortalized ovarian surface epithelium cells, primary cultures of ovarian tumors, ovarian cancer cell lines and various human placental cell types (68, 71). However, little is known about what regulates *GnRH-II* expression in these extra-pituitary cell types. In accordance with the previous finding that (Bu)2cAMP increases *GnRH-II* but not *GnRH-I* gene expression in TE671 cells (9), we found that 8-bromo cAMP enhanced human *GnRH-II* promoter activity in a wide range of cell types, including αT-3 cells, TE671 cells, OVCAR-3 cells and JEG-3 cells. Given the potential importance of GnRH-II in altering the behavior of reproductive cancer cell types, such as OVCAR-3 and JEG-3 cells (35, 68-70), we focused our attention on determining the molecular mechanisms that govern the response of the human *GnRH-II* promoter to cAMP stimulation.

We used a cAMP analogue to dissect the transcriptional mechanisms that function *via* the CRE within the human *GnRH-II* promoter in JEG-3 and OVCAR-3 cells. It is well known that cAMP regulates a diverse set of genes (220) by phosphorylating a specific sub-set of nuclear factors, such as CREB, ATF-1 and CREM (221, 222), that are all members of the basic region leucine zipper (bZIP) super-family. However, CREB is the only member of the bZIP family that is phosphorylated in response to cAMP stimulation in JEG-3 and OVCAR-3 cells (data not shown). In these cells, we found that CREB phosphorylation at Ser133 not only occurs very rapidly (within 30 min data not shown) after 8-bromo cAMP treatment, but remains elevated for up to 16 h. This appears to be an interesting cell-specific effect because a similarly rapid forskolin-induced CREB (Ser133) phosphorylation in NIH 3T3 mouse fibroblast cells is followed by a subsequent attenuation of p-CREB after 2 h *via* a protein phosphatase 1-dependent mechanism (223, 224). One possibility for this difference may be that the burst-attenuation protein phosphatase 1-dependent mechanism observed in NIH 3T3 cells is either delayed or lacking in OVCAR-3 and JEG-3 cells. Alternatively, the cAMP analogue we used

may mimic intracellular cAMP levels at higher concentrations and for longer time periods when compared to the forskolin used in the NIH 3T3 studies.

The cAMP-mediated increase in Ser133 phosphorylation of CREB is known to enhance its interaction with other nuclear proteins and their target genes *via* CREs (225). However, genome-wide analysis of CREB occupancy on target promoters by ChIP-on chip experiments suggested that less than 2% of CREB-occupied genes are responsive to a cAMP elevation (226). Interestingly, this small proportion of cAMP-responsive genes are not regulated by Ser133 phophorylated CREB alone but appear to also require the preferential recruitment of regulatory partners that promote productive interactions with co-activators (226). Thus, it is likely that cAMP-induced *GnRH-II* promoter activation involves the coordination of a multi-component complex including p-CREB and its potential co-activators. In this context, both CBP and C/EBPβ have been documented to enhance transcription through interactions with p-CREB and to facilitate activation of the basal and induced transcription machinery (24, 215, 227, 228). Phosphorylation of CREB at Ser133 also triggers the KID-mediated recruitment of the transcriptional co-activator CBP or its paralogue p300 *via* their KIX domain (229-232), which reduce the free energy required for p-CREB to bind other co-regulatory proteins (233).

The C/EBP members may form heterodimers with both bZIP and non-bZIP factors (234). For instance, CEBP/β may associate through its C-terminal region with the Q1 domain of CREB (213) or it may bind CBP/p300 (235). In addition, C/EBPβ may attract CBP to CREB, thereby creating a stronger CREB: C/EBPβ: CBP transcription complex. The relationship between C/EBPβ and CREB seems to be synergistic, enhancing the activities of both proteins. The C/EBPβ gene promoter contains a CRE motif, and its transcription can be mediated by CREB and enforced by C/EBPβ through its association with CREB (236). Our results suggest that C/EBPβ is a potential co-regulator of p-CREB that is recruited robustly to the *GnRH-II*

promoter CRE within 2 h of 8-bromo cAMP stimulation in JEG-3 and OVCAR-3 cells. By contrast, p-CREB and CBP interactions occur more progressively over a 2 -16 h time-frame, and this is reflected in a slower recruitment of CBP at the CRE region of the *GnRH-II* promoter after stimulation of the cells by 8-bromo cAMP.

The critical importance of each of these factors in mediating the 8-bromo cAMP induced increases in *GnRH-II* promoter activity in JEG-3 and OVCAR-3 cells was further demonstrated in specific knock-down experiments, and supports the concept that together they are critical components of a multi-protein complex that is assembled at the *GnRH-II* promoter CRE to mediate cAMP-signaling in these cancer cells.

Both CREB and p-CREB may bind to full-site palindromic (TGACGTCA) or half-site (CGTCA/TGACG) CREs of target genes in a cell-type dependent manner (208, 222, 226). The CRE within the *GnRH-II* promoter has been shown to modulate its expression (9), and we have demonstrated that p-CREB, CBP and C/EBPβ are all tethered at the CRE region of the *GnRH-II* promoter in OVCAR-3 and JEG-3 cells by using ChIP assays. In addition, our ChIP data indicate that loading of p-CREB onto the CRE of the *GnRH-II* promoter in un-stimulated cells is minimal, and this further supports a dynamic model for p-CREB association with the *GnRH-II* promoter after cAMP stimulation.

Recruitment of essential coactivators (such as CBP or p300) to the CREB-CRE complex is greatly enhanced by the phosphorylation of CREB (221, 237, 238). Although p-CREB and/or C/EBPβ may bind as homo- or hetero-dimers to the typical palindromic CRE motif (TGACGTCA) (239, 240), the CRE within the *GnRH-II* promoter is atypical. It is therefore possible that p-CREB and/or C/EBPβ might occupy different sites within the *GnRH-II* promoter and then undergo a physical interaction that could lead to further recruitment of other nuclear proteins such as CBP. In support of the latter possibility, p-CREB binds to an atypical CRE

within the phosphoenolpyruvate carboxykinase promoter while C/EBPβ is recruited to a separated site and then interacts with the CRE-bound p-CREB (241). Moreover, there is considerable evidence from genome-wide studies of CREB target genes (226, 242) and expression profiling of C/EBPβ target genes (243), that both proteins may recruit additional regulatory proteins to enhance transcription. This is consistent with our observation that rapid binding of p-CREB is followed by a robust association of C/EBPβ at the CRE within the *GnRH-II* promoter in OVCAR-3 and JEG-3 cells, and that this is followed by the progressive recruitment of CBP to the same site over a longer time-frame.

Taken together, our data indicate that the classical cAMP/PKA signal transduction pathway enhances the formation of a p-CREB: C/EBPβ: CBP transcription complex. This complex appears to target the CRE in the human *GnRH-II* proximal promoter and controls its activity in ovarian and placental carcinoma cells. More importantly, our data suggest that p-CREB, C/EBPβ, and CBP are recruited to the CRE of the *GnRH-II* promoter in a temporarily-defined manner to enhance its transcription in JEG-3 and OVCAR-3 cells in response to cAMP (Fig. 2.8).
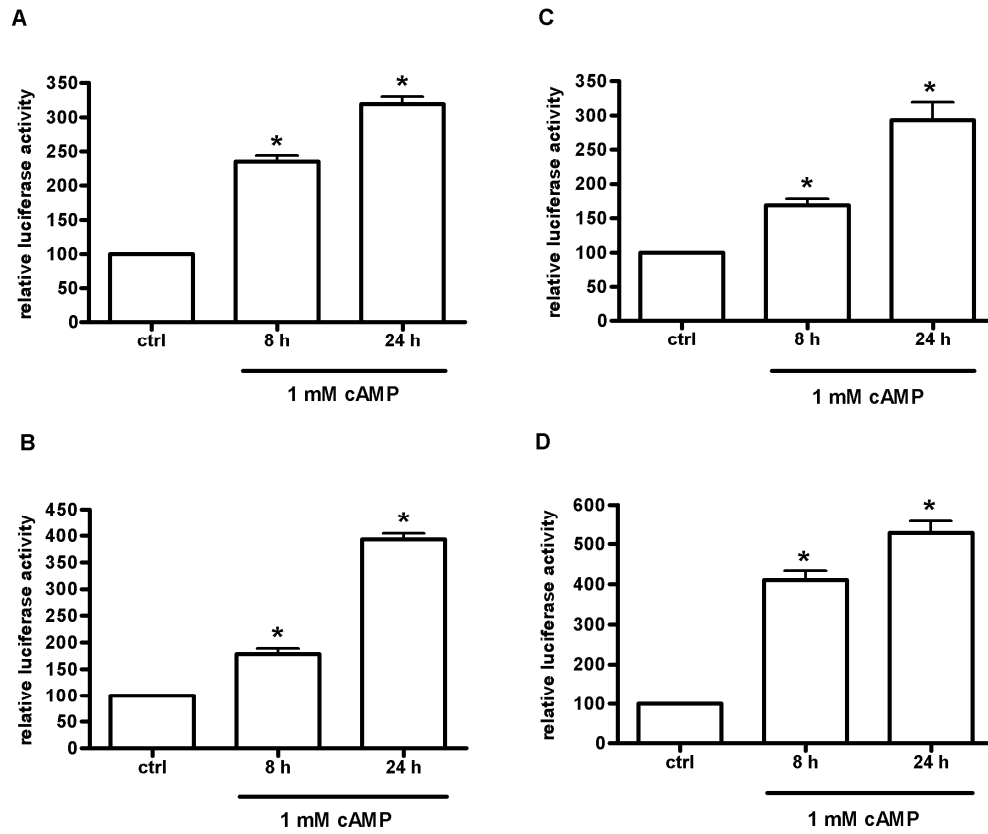
FIG. 2.1. 8-bromo cAMP induced *GnRH-II* transcriptional activity. αT-3 cells (A), TE671 cells (B), OVCAR-3 cells (C) and JEG-3 cells (D) were treated with 1 mM cAMP for 8 h and 24 h after transient transfection with a *GnRH-II* promoter luciferase construct together with a (RSV)-*lacZ* plasmid. Cell lysates were collected for luciferase assay and measurements of β-galactosidase activity as a control for transfection efficiency. Results are expressed as mean ± SEM luciferase activity/β-galactosidase activity (i.e., relative luciferase activity) of three independent experiments. * $p < 0.05$ compared to untreated control (ctrl).
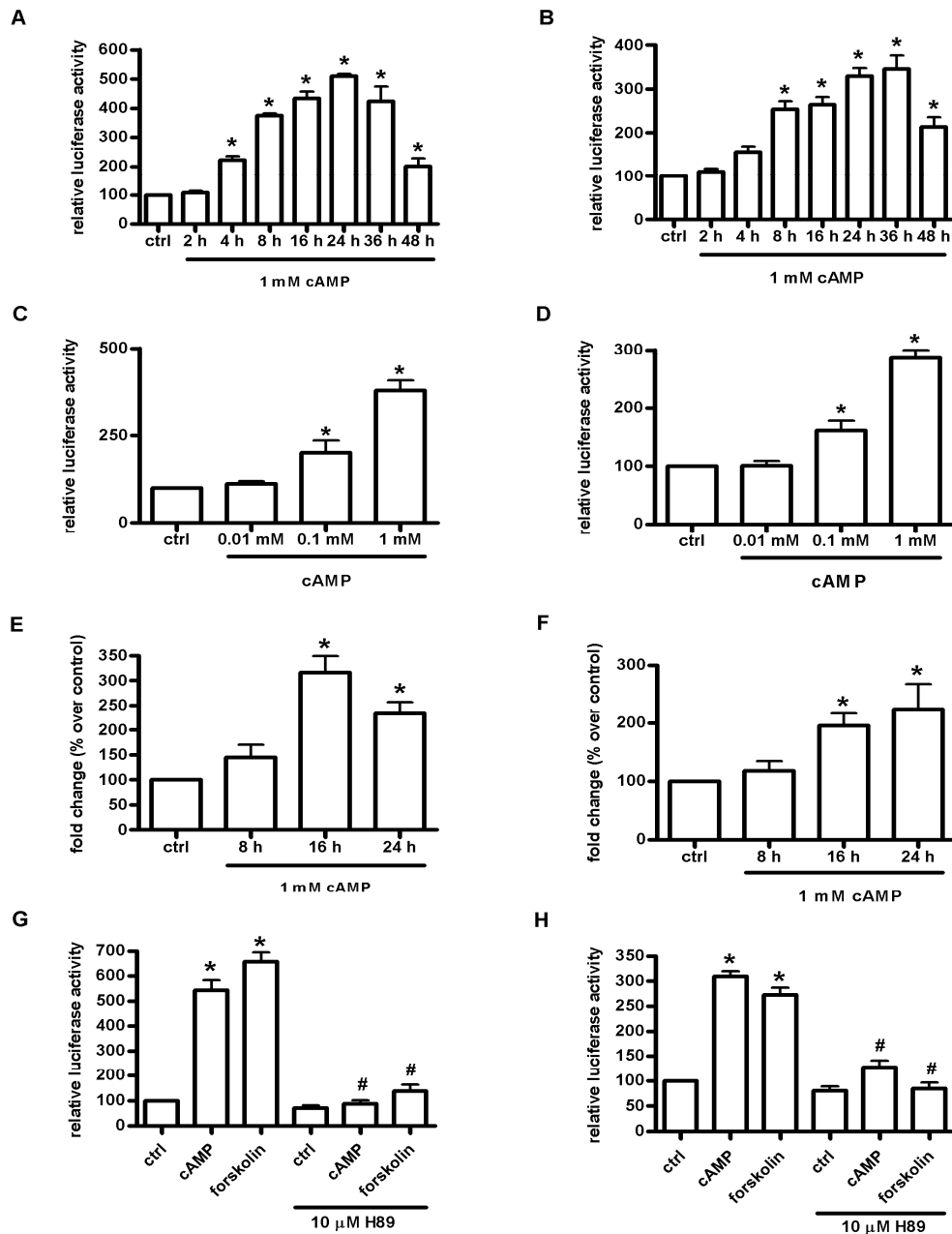
FIG 2.2. 8-bromo cAMP enhanced *GnRH-II* promoter activity and GnRH-II mRNA levels in a time-dependent manner in JEG-3 and OVCAR-3 cells. JEG-3 (A, C) and OVCAR-3 cells (B, D) were treated with 8-bromo cAMP for different times or increasing doses of cAMP (C, D) after transient transfection with a *GnRH-II* promoter luciferase construct together with a (RSV)-*lacZ* plasmid. In parallel experiments, total RNA was isolated after the administration of 8-bromo cAMP for 8 h, 16 h, 24 h and subjected for RT-qPCR to evaluate the effect of cAMP on GnRH-II mRNA levels expressed as fold changes over control (ctrl) levels in JEG-3 (E) and OVCAR-3 (F) cells. In addition, a similar experiment was performed in which the PKA inhibitor, H89, was co-treated in the presence or absence of 8-bromo cAMP or forskolin in JEG-3 (G) and OVCAR-3 (H) cells. In A, B, C, D, G and H, cell lysates were collected for luciferase assay and measurements of β-galactosidase activity as a control for transfection efficiency. Results are expressed as mean ± SEM luciferase activity/β-galactosidase activity (i.e., relative luciferase activity) of three independent experiments. * *p*<0.05 compared to untreated control (ctrl). # *p*<0.05 compared to cAMP and forskolin treated group.
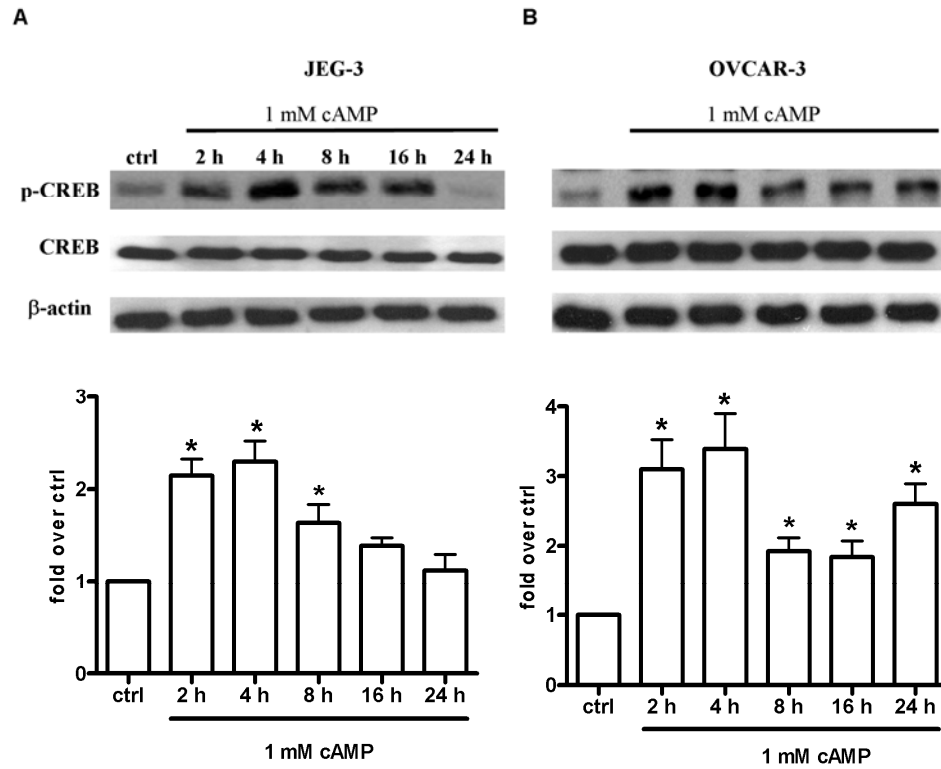
FIG. 2.3. Regulation of CREB phosphorylation at Ser133 by 8-bromo cAMP. Upper panel illustrates the levels of p-CREB, total CREB (CREB) and β-actin determined by Western blotting after the administration of 8-bromo cAMP at different time points (2, 4, 8, 16 and 24 h) in JEG-3 (A) and OVCAR-3 cells (B). The lower panel is the integrated optical density (IOD) of p-CREB levels after normalization with total CREB. Results are expressed as mean ± SEM of three independent experiments. * $p < 0.05$ compared to untreated control (ctrl).
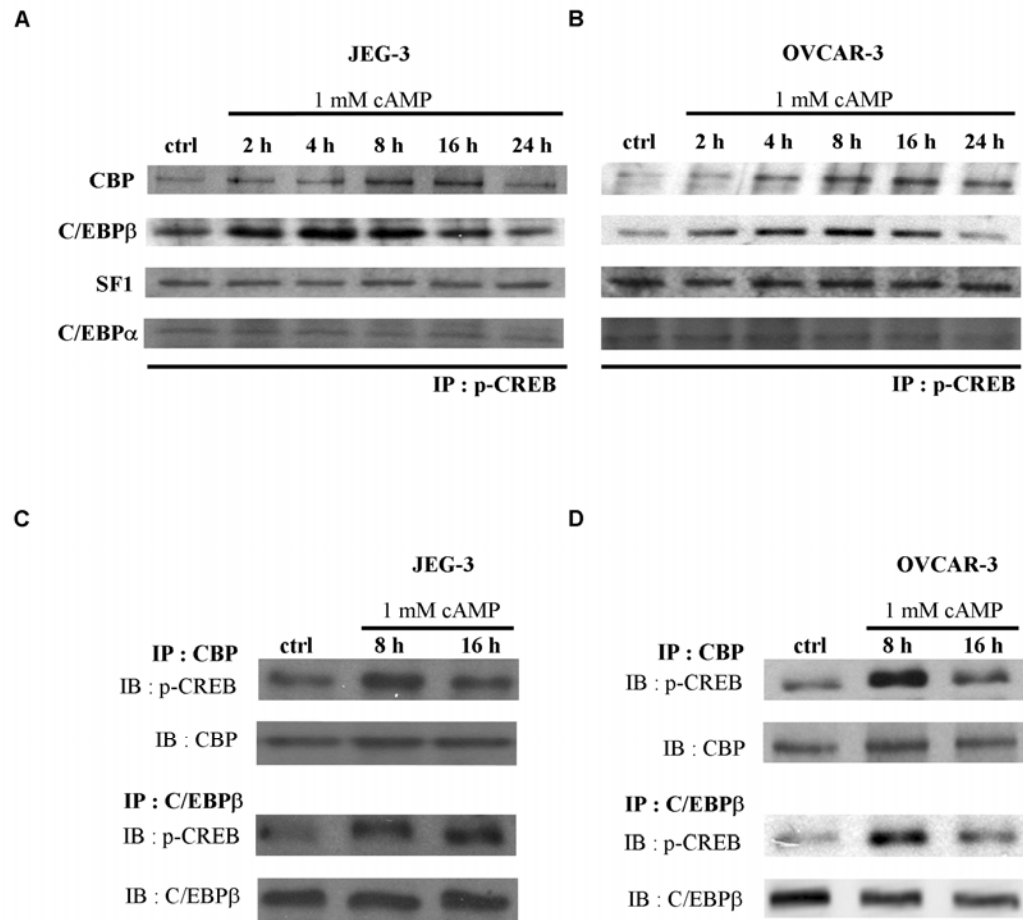
FIG. 2.4. Interaction of CBP and C/EBPβ with p-CREB increases after 8-bromo cAMP treatment. JEG-3 (A) and OVCAR-3 cells (B) were treated with 8-bromo cAMP for different times. Cell lysates were immuno-precipitated (IP) with p-CREB antibody. The immuno-precipitates were then probed with CBP, C/EBPβ, C/EBPα and SF-1 antibodies. Reciprocal immuno-precipitation (IP) was conducted upon 8-bromo cAMP treatment of JEG-3 cells (C) and OVCAR-3 cells (D) for 8 or 16 h, cell lysates were immuno-precipitated with CBP or C/EBPβ antibody, and the IPs were Western blotted probed with p-CREB antibody. Western blots are representative data of IP and reciprocal IP from three independent experiments.
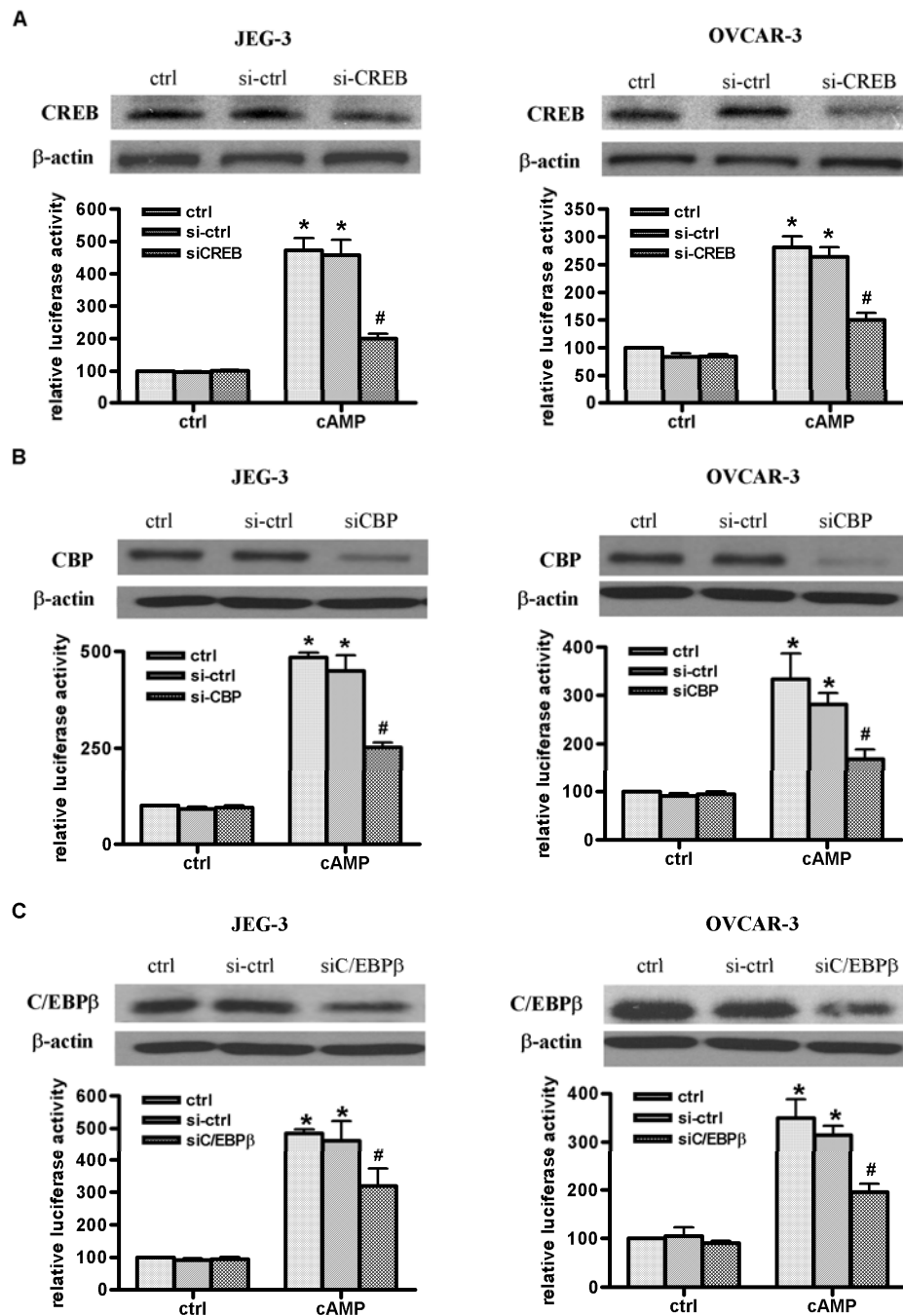
FIG. 2.5. Requirement of CREB, CBP and C/EBPβ in 8-bromo cAMP-mediated *GnRH-II* promoter activation. JEG-3 and OVCAR-3 cells were transfected with *GnRH-II*-luciferase reporter construct together with 150 nM random siRNA controls (s-ctrl) or siRNAs for CREB (A), CBP (B), C/EBPβ (C), respectively. The cells were then treated with 8-bromo cAMP for 24 h. The efficiency of the siRNA was tested by immunoblotting for CREB (67.5% knockdown), CBP (88.5% knockdown) or C/EBPβ (70% knockdown), respectively (upper panel). Cell lysates were also assayed for luciferase activity and measurements of β-galactosidase activity as a control for transfection efficiency, the result of which are expressed as mean ± SEM luciferase activity/β-galactosidase activity (i.e., relative luciferase activity) of three independent experiments. * *p*<0.05 compared to cells treated with an siRNA control (si-ctrl). # *p*<0.05 compared to cells treated with respective siRNAs and followed by 8-bromo cAMP treatment.

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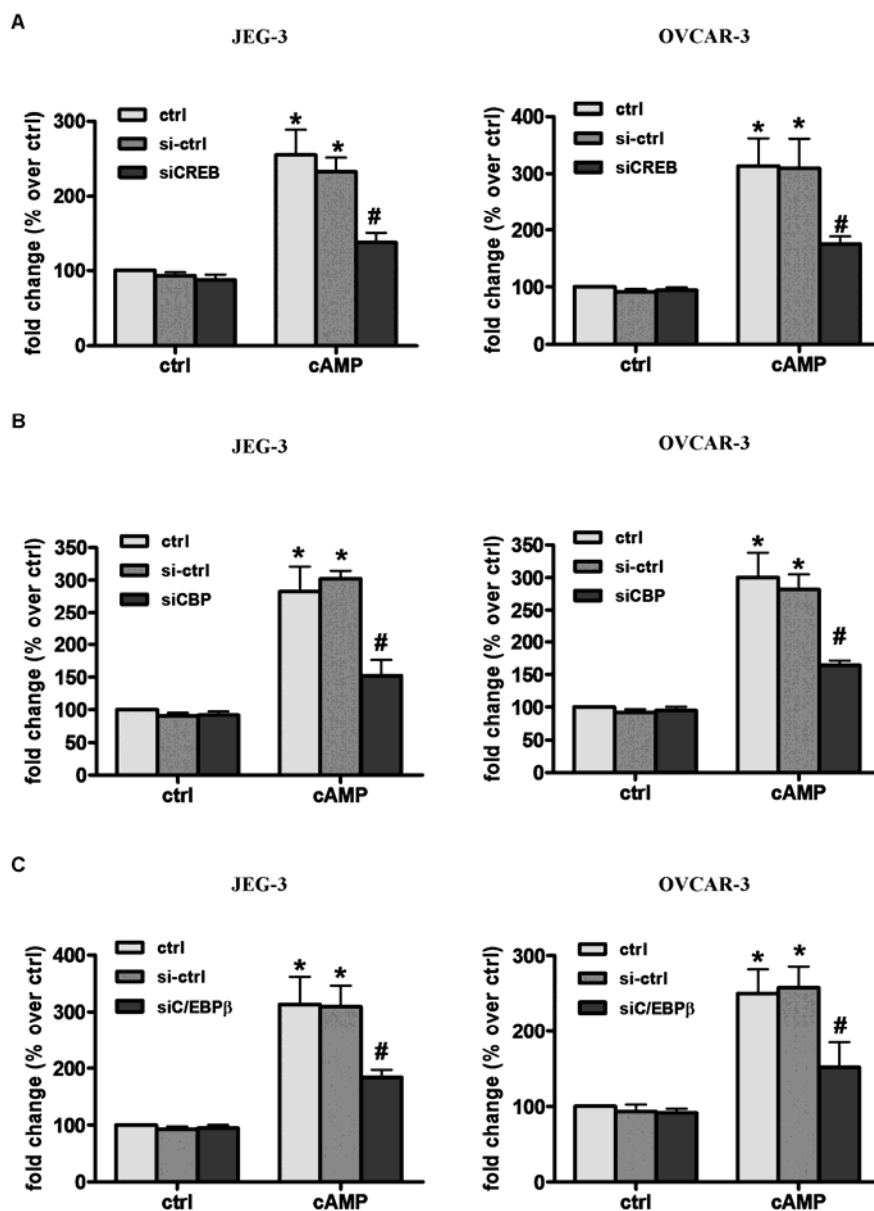
FIG. 2.6. Involvement of CREB, CBP and C/EBPβ in 8-bromo cAMP-mediated changes in GnRH-II mRNA levels in JEG-3 and OVCAR-3 cells. Cells were transfected with 150 nM random siRNA controls (si-ctrl) or siRNAs for CREB (A), CBP (B), C/EBPβ (C), respectively, and then treated with 8-bromo cAMP for 16 h. The efficiency of the siRNAs was tested by immunoblotting for CREB (67.5% knockdown), CBP (88.5% knockdown) or C/EBPβ (70% knockdown), respectively (Fig. 5). Total RNA was isolated and cDNA was used in RT-qPCR to evaluate the effect of cAMP on GnRH-II mRNA levels expressed as fold changes over control (ctrl) levels in untreated JEG-3 and OVCAR-3 cells. * $p<0.05$ compared to cells treated with a siRNA control (si-ctrl). # $p<0.05$ compared to cells treated with respective siRNAs and followed by 8-bromo cAMP treatment.
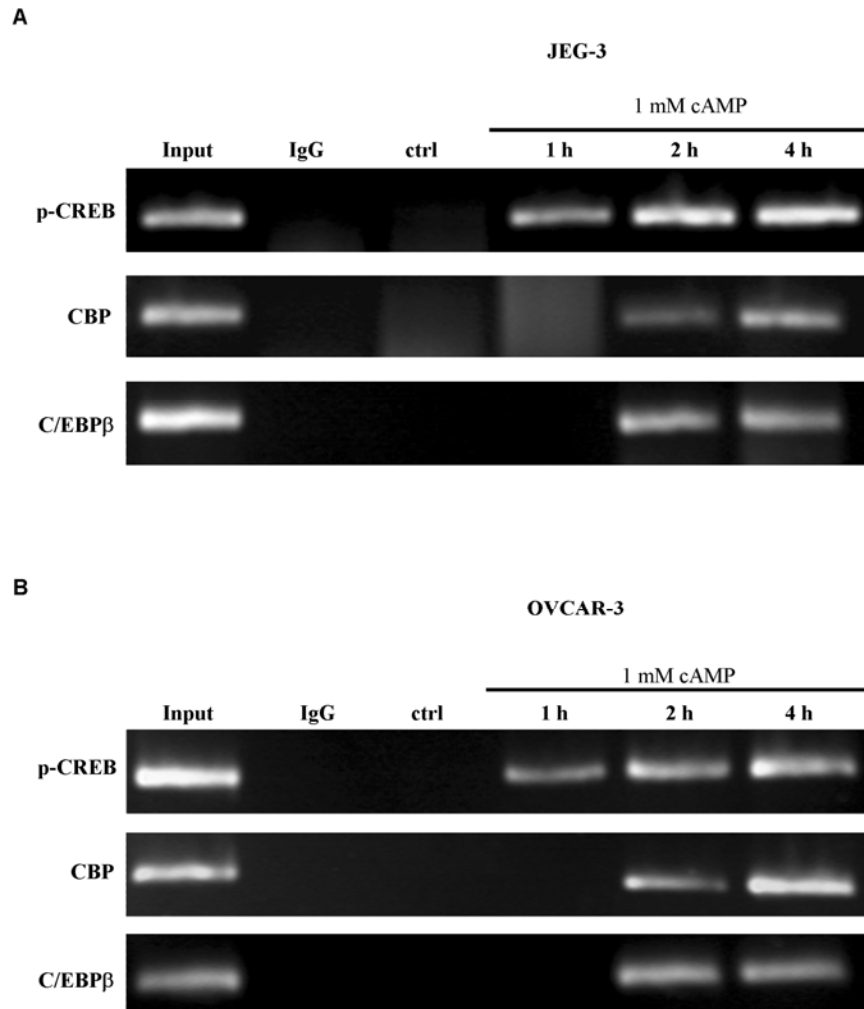
FIG. 2.7. Association of p-CREB, C/EBPβ and CBP with the CRE region of *GnRH-II* promoter is increased by 8-bromo cAMP treatment. Chromatin immuno-precipitation (ChIP) analysis was performed as described in *Materials and Methods*. JEG-3 (A) and OVCAR-3 cells (B) were treated with 1 mM 8-bromo cAMP for 1 h, 2 h or 4 h, or were untreated (ctrl). Cross-linked, sheared chromatin was immuno-precipitated (IP) with p-CREB, C/EBPβ or CBP antibodies, and recovered chromatin was subjected to PCR analysis using primers spanning the CRE region of the *GnRH-II* promoter. The IgG lanes are ChIPs performed using non-specific IgG. An ethidium bromide-stained gel of PCR products shows a representative of ChIP analysis from three independent experiments.
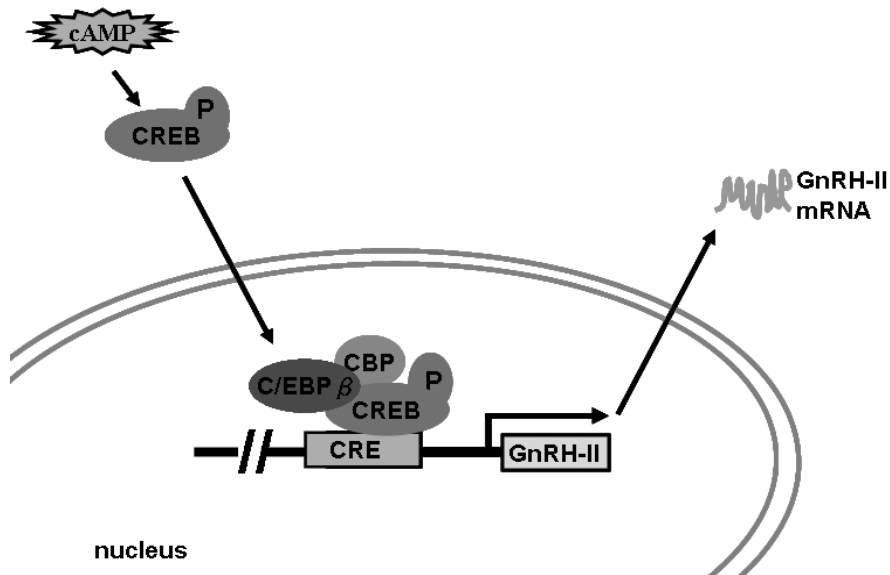
FIG 2.8. Proposed model for CRE-mediated *GnRH-II* gene transcription. The classical cAMP/PKA signal transduction pathway enhances the formation of a p-CREB: C/EBPβ: CBP transcription complex. This complex appears to target the CRE in the human *GnRH-II* proximal promoter and controls its activity in ovarian and placental carcinoma cells. p-CREB, phosphorylated cAMP responsive binding protein; C/EBPβ, CCAAT/enhancer binding protein beta; CBP, CREB binding protein; CRE, cAMP responsive element.

CHAPTER 3. Epidermal growth factor induced GnRH-II synthesis contributes to ovarian cancer cell invasion[2]

3.1 Introduction

The two gonadotropin-releasing hormones (GnRH-I and GnRH-II) and the GnRH receptor (GnRHR) have been detected in human ovarian surface epithelial cells and ovarian cancer cell lines (48), and these GnRH subtypes regulate the growth (58, 66, 70) and metastatic activity (71, 78) of ovarian cancer cells. Despite advances in our knowledge of the functional role of GnRH-II in ovarian cancer, the endocrine regulation of GnRH-II expression in ovarian cancer cells is poorly understood. In female reproductive tumor cell lines, studies of cell signaling have focused on the cAMP-mediated activation of protein kinase A (PKA) and the subsequent phosphorylation-dependent activation of CREB (53). Elevated phosphorylated CREB (p-CREB) recruits the co-regulators, CREB binding protein (CBP) and CCAAT/enhancer binding protein β (C/EBPβ), and increases the cAMP responsive element (CRE)-dependent expression of the *GnRH-II* gene in a coordinated and temporally-defined manner (53). Additional *cis*-regulatory regions, including a minimal promoter region that includes two enhancer elements (E-boxes); an ETS-like element in the un-translated exon 1(10), and a NFκB recognition site in the first intron (12) of the human *GnRH-II* gene, have also been identified.

In ovarian carcinoma cells, EGF and EGF-related peptides function as autocrine growth factors (244). The EGF receptor (EGFR) also plays a role in cancer cell biology and is a key therapeutic target in ovarian cancer (122). Classical EGFR signal transduction is initiated by ligand binding to its extracellular domain, which leads to a conformational change in the receptor and induces its homodimerization or heterodimerization with other EGFR family

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[2] A version of this chapter has been published. **Poon SL**, Hammond GT, Leung PC 2009 Mol Endocrinol, 23(10):1646-56.

members (130, 245). Ligand-induced EGFR dimerization allows trans-phosphorylation of specific tyrosine residues that serve as docking sites for intracellular signaling molecules (122), thereby stimulating the receptor's intrinsic tyrosine kinase activity (246). Each EGF receptor is capable of recruiting a specific subset of adapter proteins and signaling molecules, such as Ras/Raf1/mitogen-activated protein (MAP) kinase and PI3K/Akt, which subsequently activate downstream mediators to stimulate cell proliferation, invasion and angiogenesis (129-131). Gene amplification, genetic mutation, and altered transcription or translation result in aberrant EGFR expression that contributes to malignant transformation (247, 248), poor prognosis and decreased therapeutic responsiveness in ovarian cancer patients (120, 249, 250). Thus, anticancer agents targeting the EGFR or its downstream signaling/target genes hold great promise.

In the present study, we sought to determine whether EGF regulates the expression of GnRH-II in ovarian cancer cells. Our results demonstrate that EGF treatment of OVCAR-3 cells increases *GnRH-II* promoter activity and GnRH-II mRNA levels through the auto-phosphorylation of EGFR and the activation of ERK1/2/p-CREB/C/EBPβ signaling. The stimulatory effect of EGF was observed in three human ovarian cancer cell lines (OVCAR-3, CaOV-3 and SKOV-3 cells), and this increase in GnRH-II by EGF promotes the EGF-induced invasiveness of ovarian cancer cells, suggesting that GnRH-II is a novel downstream target of EGF in ovarian cancer cell tumorigenicity.

3.2 Materials and methods

Cells and cell culture

The human ovarian adenocarcinoma cell lines, OVCAR-3, CaOV-3 and SKOV-3 were obtained from Ameriacan Type Culture Collection (Manassas, VA, USA). The cells were maintained in M199/MCDB105 (Invitrogen Inc., Burlington, ON, Canada) supplemented with

10% fetal bovine serum (FBS; Hyclone Laborataries Inc., Logan, UT, USA). Cultures were maintained at 37 C in a humidified atmosphere of 5% CO_2. The cells were sub-cultured when they reached about 90% confluence using a trypsin/EDTA solution (0.05% trypsin, 0.5 mM EDTA).

Antibodies and reagents

The polyclonal β-actin antibody and polyclonal C/EBPβ antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The monoclonal phospho-ERK1/2 (Thr202/Tyr204) antibody, polyclonal total ERK1/2 antibody, polyclonal total EGFR and monoclonal phosphor-CREB (Ser133) antibody were obtained from Cell Signaling Technology (Danvers, MA). The monoclonal GnRHR antibody was obtained from Neomarkers (Fremont, CA), horseradish peroxidase-conjugated goat anti-mouse IgG and goat anti-rabbit IgG were obtained from Bio-Rad Laboratories (Hercules, CA). Horseradish peroxidase-conjugated donkey anti-goat IgG was obtained from Santa Cruz. GnRH-II analog (DArg6-Azagly10-GnRH-II) was purchased from Bachem (Belmont, CA). Human epidermal growth factor (EGF) and EGFR inhibitor (AG1478) were obtained from Sigma. MAPK inhibitor (PD98059 and U0126) was obtained from Calbiochem (San Diego, CA).

Plasmid construction and reporter gene assays

The *GnRH-II* promoter-driven luciferase reporter gene construct was generated by PCR amplification of human genomic DNA using sequence-specific primers designed to amplify 2 kb upstream of the 5′ flanking region in *GnRH-II* promoter and followed by its subsequent cloning into the promoter-less pGL2-Basic vector (Promega, Madison, WI, USA) (10). Mutation of the CRE within the *GnRH-II* promoter was generated using the *GnRH-II* promoter-driven luciferase construct as template by the Quickchange II XL site directed mutagenesis kit

(Stratagene, La Jolla, CA, USA), and the following oligonucleotide primers: forward, 5´-CTCTCTTCCCCTCTGA<u>AGA</u><u>*TAC*</u><u>CA</u>CTGGAGTCTGGGGGTG and reverse, 5´-CACCCCCAGACTCCAGT<u>G</u><u>*GTA*</u><u>TCT</u>TCAGAGGGGAAGAGAG. The product was sequenced to verify that only the desired mutation had occurred during the mutagenesis reaction.

Transient transfections were carried out using Lipofectamin 2000 Reagent (Invitrogen Inc., Burlington, ON, Canada) following the manufacturer's protocol. To correct for transfection efficiencies, the Rous sarcoma virus (RSV)-*lacZ* plasmid was co-transfected into the cells with the *GnRH-II* promoter-driven luciferase reporter gene construct. Briefly, 5×10^5 cells were seeded into six-well tissue culture plates the day before transfection. The *GnRH-II* promoter-driven luciferase reporter gene construct (1 μg) and 0.5 μg RSV-*lacZ* plasmid were co-transfected into cells grown in standard culture medium containing FBS. In some experiments, 150 nM siCREB or a control siRNA oligonucleotide (Qiagen Inc., Mississauga, ON, Canada) were co-transfected with the reporter plasmids. After 6 h, 2 ml of serum free medium was added and the cells were further incubated overnight (18 h). The culture medium was then removed and the cells were treated with EGF or 8-bromo cAMP, respectively in serum free medium for the times indicated. Cellular lysates were collected with 150 μl reporter lysis buffer (Promega) and assayed for luciferase activity. The β-Galactosidase Enzyme Assay System (Promega) was used to measure expression from the (RSV)-*lacZ* plasmid, and promoter activities were expressed as luciferase activity/β-galactosidase activity.

Reverse transcription quantitative real-time PCR (RT-qPCR)

After treatment with 8-bromo cAMP, medium was removed from the culture dish and RNA was extracted using Trizol (Invitrogen). The RNA concentration was measured based on the absorbance at 260 nm, and its integrity was confirmed by agarose-formaldehyde gel electrophoresis. Total RNA (2.5 μg) was reverse-transcribed into cDNA using a first-strand

cDNA synthesis kit (GE Healthcare Bio-Science, Piscataway NJ, USA) following the manufacturer's procedure. The primers used for RT-qPCR were designed using Primer Express Software v2.0 (Applied Biosystems, Foster City, CA, USA). The primers for GnRH-II mRNA are: sense, 5´-CTGCTGACTGCCCACCTT; and antisense, 5´-GCTTTCCTCCAGGGTACCAG. The primers for GAPDH are: sense, 5'-GAGTCAACGGATTTGGTCGT; and antisense, 5´-GACAAGCTTCCCGTTCTCAG. The reactions were set up with 16.5 µl SYBR Green PCR Master Mix (Applied Biosystems). All RT-qPCR experiments were run in triplicate and a mean value was used for the determination of mRNA levels. Negative controls containing water instead of sample cDNA were used in each experiment. Relative quantification of the mRNA levels of GnRH-II was performed using the comparative Cq method with GAPDH as the reference gene and with the formula $2^{-\Delta\Delta Cq}$.

Nuclear protein extraction, Western blotting and immunoprecipitation

Briefly, cells were washed with ice-cold PBS and harvested with 1 ml solution A (10 mM Hepes, pH 7.9, 10 mM KCl, 10 mM EDTA, 0.5 mM dithiothreitol, 1 µg/ml aprotinin, and 1 µg/ml protein inhibitor cocktail). Cell lysates were transferred to 1.5 ml centrifuge tubes and placed in an orbital rocker for 10 min at 4 C. Nuclear pellets were obtained by centrifugation at 14,000 g at 4 C for 10 min, and supernatants were collected for cytoplasmic protein. Nuclear pellets were re-suspended in solution B (100 mM HEPES, pH 7.9, 2 M NaCl, 5 mM EDTA, 50% Glycerol) and placed in an orbital rocker for 2 h at 4 C. After centrifugation at 14,000 g at 4 C for 5 min, supernatants containing the nuclear protein extracts were removed. The nuclear extracts were then subjected to electrophoresis on an 8% SDS-PAGE gel and Western blotted for detection with appropriate antibodies.

Immunoprecipitation was conducted as follows: nuclear extracts were incubated with p-CREB antibody (10 ug/ml) followed by the antibody capture affinity ligand provided by the

immunoprecipitation kit (Upstate, MA, USA) at 4 C overnight. The immuno-precipitated proteins were then subjected to electrophoresis on an 8% SDS-PAGE gel and detected with appropriate antibodies after Western blotting.

Chromatin immuno-precipitation (ChIP)

All reagents, buffers and supplies were included in a ChIP-IT^TM kit (Active Motif, Inc., Carlsbad, CA). Briefly, the cells were cross-linked with 1% formaldehyde for 10 min at room temperature. After washing and treatment with glycine Stop-Fix solution, the cells were re-suspended in lysis buffer and incubated for 30 min on ice. The cells were homogenized and nuclei were re-suspended in shearing buffer, and subjected to pre-optimized ultrasonic disruption conditions to yield 100-500 bp DNA fragments. The chromatin was pre-cleared with Protein G beads and incubated (overnight at 4 C) with 1 μg of the following antibodies: negative control mouse IgG (Active Motif), p-CREB antibody (Cell Signaling). Protein G beads were then added to the antibody/chromatin incubation mixtures and incubated for 1.5 h at 4 C. After extensive washing, the immuno-precipitated DNA/protein complex was removed from the beads by elution buffer. To reverse cross-links and remove RNA, 5 M NaCl and RNase were added to the samples and incubated at 65 C for 4 h. The samples were then treated with proteinase K for 2 h at 42 C and the DNA was purified using gel exclusion columns. The purified DNA was subjected to PCR amplification (1 cycle of 94 C for 3 min; 40 cycles of 94 C for 20 sec; 64 C for 30 sec and 72 C for 30 sec) for the CRE site (-860/-853 bp) within the *GnRH-II* promoter using specific forward, 5´-CCAGCCTAAAGCAAGAGTCC and reverse, 5´-GTCTATAAATCCTGGGGCCA primers. As an input control, 10% of each chromatin preparation was used. The PCR products (213 bp) were resolved by electrophoresis in a 2.5% agarose gel and visualized by ethidium bromide staining (53). The ChIP assay was performed at least three times, and consistent data were obtained between experiments.

Invasion assay

The invasion assay was performed in Boyden chambers with minor modifications (251). Filters (8 μm pore size, 24 wells, BD Biosciences) were coated with 40μL of 1 mg/ml growth-factor reduced Matrigel (BD Biosciences). Cells in M199/MCDB105 medium supplemented with 0.1% FBS were incubated for 48 h against a gradient of 10% FBS for OVCAR-3 cells and 5% FBS for CaOV-3 and SKOV-3 cells. Cells that penetrated the membrane were fixed with ice-cold methanol, stained with Hoechst 33258 (Sigma), and the number of nuclei was counted using Northern Eclipse 6.0 software from Empix Imaging (Mississauga, ON, Canada). Each treatment was done in duplicate and five microscopic fields were counted per Boyden chamber.

Data analysis

Reporter gene assays and real time PCR data are shown as the mean ± SEM of three independent experiments. Data were analyzed by one-way ANOVA, followed by Tukey test using the computer software PRISM (GraphPad Software Inc., San Diego, CA, USA). Values were considered significantly different from each other at $p < 0.05$.

3.3 Results

EGF stimulates GnRH-II expression in OVCAR-3, CaOV-3 and SKOV-3 cells

When treated with EGF, 2-3 fold increases in GnRH-II mRNA levels were observed in OVCAR-3 (Fig. 3.1A), CaOV-3 (Fig. 3.1B) and SKOV-3 (Fig. 3.1C) cells. While EGF specifically induced the expression of GnRH-II in these cell lines, it had no effect on GnRH-I or GnRHR mRNA levels after 24 h treatment. However, by treating all three cell lines with EGF for 2, 4, 8, 16, and 24 h, we observed a modest increased in GnRHR protein levels after 8 h treatment in OVCAR-3 and CaOV-3 cells, but not in SKOV-3 cells (Fig. 3.1D). In addition, increased GnRHR levels returned to the pre-treatment levels after 24 h in both OVCAR-3 and

CaOV-3 cells (Fig. 3.1D). Using OVCAR-3 cells as our model, EGF treatment increased the activity of a *GnRH-II* promoter-driven luciferase reporter gene in a concentration-dependent manner (Fig. 3.2A). Blocking downstream signaling by using the EGF receptor inhibitor, AG1478, abolished the EGF-induced *GnRH-II* promoter-driven luciferase reporter gene activity (Fig. 3.2B), as well as GnRH-II mRNA levels in OVCAR-3 cells (Fig. 3.2C), suggesting that the effect of EGF on GnRH-II regulation is EGF receptor-dependent.

EGF induces the MAPK pathway to enhance phosphorylation of CREB and its interaction with C/EBPβ upon EGFR activation

Treatment of OVCAR-3 cells with EGF induced phosphorylation of EGFR at tyrosine 992 and tyrosine 1045 (Fig. 3.3A) but not at tyrosine 1068 (data not shown). In addition, EGF very rapidly induces the phosphorylation of ERK1/2 with a maximum response at 15 min (Fig. 3.3B), suggesting that MAPK signaling may be involved in the regulation of GnRH-II expression in OVCAR-3 cells. The transcription factor CREB is a target of MAPK signaling (252), and is necessary for the regulation of GnRH-II gene expression (53). Western blot results indicate that CREB phosphorylation not only occurs very rapidly (within 10 min) but remains elevated for up to 8 h (Fig 3.3C). Thus, we examined whether EGF-induced MAPK signaling results in the phosphorylation of CREB. We used a pharmacological inhibitor (PD98059) to block EGF-induced ERK1/2 signaling and examined the status of p-CREB (Fig 3.3D). The results of these Western blotting experiments indicate that EGF rapidly induced CREB phosphorylation within 2 h, and that pre-treatment with PD98059 attenuated EGF-induced phosphorylation of CREB in OVCAR-3 cells (Fig. 3.3D).

Previously, we demonstrated that an increase of p-CREB recruits C/EBPβ and up-regulates GnRH-II transcription (53). Using immunoprecipitation, we have shown that the administration of 100 ng/ml EGF to OVCAR-3 cells enhances the association of p-CREB with

C/EBPβ (Fig. 3.3E). More importantly, the EGF-induced interaction between p-CREB and C/EBPβ was markedly reduced upon pre-treatment with U0126, a pharmacological agent that specifically inhibits p-ERK1/2 signal transduction pathways (Fig. 3.3E).

EGF induced phosphorylation of CREB activates the cAMP responsive element (CRE) within the GnRH-II promoter

To determine whether EGF-induced p-CREB in OVCAR-3 cells targets the CRE region within the *GnRH-II* promoter, a chromatin immuno-precipitation (ChIP) assay was performed. Cross-linked, sheared chromatin from EGF-treated OVCAR-3 cells was immuno-precipitated with p-CREB antibody, and the recovered DNA was subjected to PCR using primers specific to the CRE region (-860/-853) of the *GnRH-II* promoter. As shown in figure 4A, a 213 bp PCR product was amplified from p-CREB-immuno-precipitated DNA samples in OVCAR-3 cells treated with EGF, and this was already evident after 1 h, and is increased at 2-4 h of EGF stimulation (Fig. 3.4A). By contrast, little or no PCR product was observed with DNA recovered when control IgG was used for the immuno-precipitation or cells untreated with EGF. These data reveal a specific association between p-CREB at the CRE region of the *GnRH-II* promoter in OVCAR-3 cells after treatment with EGF and that this occurs in a temporally-defined manner.

A specific siRNA oligonucleotide was used to knock down endogenous CREB levels to verify its involvement in *GnRH-II* expression in OVCAR-3 cells (Fig. 3.4B). In this experiment, EGF induced *GnRH-II* promoter activity in cells co-transfected with control siRNA, while co-transfection with CREB specific siRNA compromised this effect of EGF (Fig. 3.4C). To further verify that the CRE within the *GnRH-II* promoter is sufficient for EGF-regulated *GnRH-II* expression, we mutated 3 bp within the CRE (wild type CRE: agacgtca; mutated CRE: aga*tac*ca) of the *GnRH-II* promoter-driven luciferase reporter gene. Transfection of the mutated reporter

gene in OVCAR-3 cells resulted in a 35% decrease in basal *GnRH-II* promoter activity. Moreover, while treatment with 1mM cAMP led to a significant increase in the wild-type *GnRH-II* promoter-driven luciferase reporter gene, it had no effect in cells transfected with the CRE-mutated version of the same reporter construct. In a parallel experiment, 100 ng/ml EGF induced the activation of the wild-type *GnRH-II* promoter, but this stimulation was reduced by 70% when we mutated the CRE within the *GnRH-II* promoter (Fig. 3.4D). These results indicate that the CRE we have examined is important for basal *GnRH-II* promoter activity, and that EGF exerts most of its effects through this particular *cis*-acting element to enhance the transcription of *GnRH-II* in OVCAR-3 cells.

EGF and GnRH-II act additively to enhance ovarian cancer cell invasion

As a strong mitogen, EGF enhances cell motility and induces secretion of proteolytic enzymes to increase the invasiveness in ovarian cancer cells (24, 253). To evaluate the effect of EGF-induced *GnRH-II* expression in ovarian cancer cells, OVCAR-3 cells, CaOV-3 cells and SKOV-3 cells were treated with 100 ng/ml EGF or 10 nM GnRH-II for 24 h prior to an invasion assay. In these experiments, EGF-treated cells exhibited increased invasiveness as compared with their untreated controls. Interestingly, in the GnRH-II treated group, only OVCAR-3 cells (Fig 3.5A) and CaOV-3 cells (Fig. 3.5B) showed 20 fold and 2 fold increases in their invasiveness, respectively, as compared to the controls, whereas SKOV-3 cells did not respond (Fig. 3.5C). When we used Western blotting to check the expression of the GnRHR in all three cell lines, we found that the expression of GnRHR in SKOV-3 cells is lower than in OVCAR-3 and CaOV-3 cells (Fig. 3.5D), and this suggests that ovarian cancer cells respond to GnRH-II treatment in relation to their GnRHR content. More importantly, to evaluate whether GnRH-II acts in concert with EGF to enhance the invasiveness of ovarian cancer, we co-treated all three cell lines with EGF and GnRH-II, and the results imply that these two agents have an additive

effect on the invasiveness of OVCAR-3 (Fig. 3.5A) and CaOV-3 cells (Fig. 3.5B), while GnRH-II has no additive effect on the EGF-induced invasiveness in SKOV-3 cells (Fig. 3.5C). In addition, we confirmed that the expression of GnRHR was not regulated by GnRH-II (Fig. 3.5E) treatment but found that a small but consistent transient increase in GnRHR levels after EGF treatment only in OVCAR-3 and CaOV-3 cells (Fig. 3.1D), suggesting that EGF not only up-regulates GnRH-II mRNA levels but also transiently increases the GnRHR levels, which would likely further enhance EGF/GnRH-II-induced invasion in ovarian cancer cells.

EGF-induced GnRH-II production enhances the invasiveness of ovarian cancer cells

To further explore the possibility that EGF-induced synthesis of GnRH-II acts in an autocrine manner to increase the invasiveness of ovarian cancer cells, a siRNA approach used to knock down the endogenous levels of GnRHR in OVCAR-3 cells and CaOV-3 cells. The transfected cells were then treated with 100 ng/ml EGF or 10 nM GnRH-II for 24 h prior to an invasion assay. This demonstrated that depletion of GnRHR in OVCAR-3 (Fig. 3.6A) and CaOV-3 (Fig. 3.6B) cells inhibited the GnRH-II induced invasion as compared with cells transfected with a scrambled siRNA control. More importantly, the siRNA-mediated knock down of GnRHR levels in these two cell lines also partially abolished EGF-induced invasion, further confirming that GnRH-II/GnRHR signaling is involved in the EGF-induced invasion of OVCAR-3 and CaOV-3 cells.

3.4 Discussion

It is becoming increasingly apparent that GnRH-II acts as an autocrine/paracrine regulator in non-pituitary tissues in addition to its role in the regulation of gonadotropin synthesis and steroid hormone production (52, 177), and is an important player in cancer cell biology (71). Ovarian cancer cells also express the EGFR, and EGF is a critical mitogen

involved in the differentiation of normal ovarian surface epithelial cells and the motility of ovarian cancer cells (47, 129). In the present studies, we demonstrate for the first time that *GnRH-II* expression is regulated by EGF activation of its receptor and the ERK1/2/p-CREB/C/EBPβ intracellular signaling pathway in an ovarian cancer cell model. More importantly, we have obtained evidence that EGF–stimulated *GnRH-II* expression constitutes a specific autocrine/paracrine loop that contributes to ovarian cancer motility.

As observed by others (168, 254, 255), EGFR activation in OVCAR-3 cells stimulates the classical MAPK/ERK1/2 pathways. Treatment of OVCAR-3 cells with EGF elicits the autophosphorylation of the EGFR at tyrosine 992 and tyrosine 1045, whereas tyrosine 992 is a target of the MAP kinase cascade. It is known that activation of the MAP kinase cascade and the subsequently phosphorylation and translocation of ERK1/2 activates transcription factors, including NFκB, HIF-1α and CREB, resulting in increases in the transcription of pro-invasive genes such as VEGF or COX-2 (3, 256, 257). In our experiments, we have found that pre-treatment of OVCAR-3 cells with the EGFR inhibitor, AG1478, abolishes EGF/EGFR downstream signaling, and that this inhibits the EGF-induced activation of a *GnRH-II* promoter and increases GnRH-II mRNA levels. Interestingly, EGF treatment of OVCAR-3 cells leads to increased ERK1/2 phosphorylation for up to 2 h, and others have noted that such a prolonged phosphorylation of ERK1/2 results in its increased nuclear retention (258-260). Thus, it is plausible that EGF treatment of OVCAR-3 cells prolongs the nuclear retention of activated ERK, and leads to an up-regulation of *GnRH-II* expression in ovarian cancer cells.

It is widely accepted that CREB plays a critical role in *GnRH-II* expression through the transcriptional activation of the *GnRH-II* promoter (53). In this study, we present evidence that EGF treatment causes phosphorylation of CREB at serine133 and increases its interaction with C/EBPβ, and that these steps are required for recruitment of the transcriptional co-activator CBP (227, 261), as well as the transcriptional activation of the *GnRH-II* promoter. In OVCAR-3 cells,

treatment with EGF enhanced CREB phosphorylation for up to 8 h, and this is correlated with the loading of p-CREB at the CRE within *GnRH-II* promoter, and an increase in *GnRH-II* promoter activity and GnRH-II mRNA levels, which are both maximal at 16 h ~ 24 h. It is also known that the phosphorylation of ERK can lead to the activation of RSK and MSK signaling and thereby stimulate the translocation of phosphorylated CREB (252) or phosphorylated C/EBPβ (262, 263). It was therefore of interest that blockade of EGF-induced ERK1/2 activation with a selective MAPK inhibitor (PD98059) was sufficient to block the phosphorylation of CREB, as demonstrated by Western blotting, as well as the transcriptional activation of a *GnRH-II* promoter-driven luciferase reporter gene (data not shown) in OVCAR-3 cells. In addition, treatment with another potent ERK1/2 inhibitor (U0126) markedly reduced the EGF-induced interaction of p-CREB with C/EBPβ in OVCAR-3 cells. Thus, it appears that ERK activation is required for the phosphorylation of CREB and its interaction with C/EBPβ, and this will contribute to its subsequent effects on the transcriptional activation of the *GnRH-II* gene after treatment of ovarian cancer cells with EGF.

The *GnRH-II* gene is regulated by several cis-acting elements within its promoter sequence (2), including an CRE (agacgtca) at nucleotide sequence -860 to -853 bp relative to the translation start site in the *GnRH-II* promoter, which responds to cAMP analogs in human TE671 neuroblastoma cells (9) and human reproductive cancer cells (53). This CRE from the *GnRH-II* promoter has been shown to bind p-CREB/CBP/C/EBPβ under 8-bromo cAMP stimulation (53). Currently, our studies suggest that this CRE is one of the key *cis*-acting elements that respond to EGF stimulation. This was verified when this CRE was mutated within the *GnRH-II* promoter, and resulted in a blockade of EGF-induced *GnRH-II* promoter activity. Furthermore, we used a ChIP assay to demonstrate the mechanisms of EGF-regulated CRE-mediated effects within the *GnRH-II* promoter. In this context, EGF-induced tethering of p-CREB at the CRE region of the *GnRH-II* promoter in OVCAR-3 cells, while loading of p-

CREB onto the CRE of the *GnRH-II* promoter in un-stimulated cells was minimal: a finding that supports a dynamic model for p-CREB association with the *GnRH-II* promoter after EGF stimulation. The critical importance of p-CREB in mediating EGF-induced increases in *GnRH-II* promoter activity in OVCAR-3 cells was further demonstrated in specific knock-down experiments, which further support the concept that p-CREB is a critical component that assembles at the *GnRH-II* promoter CRE after the EGF treatment of OVCAR-3 cells.

It is known that EGF and EGF-like peptides including transforming growth factor-α and amphiregulin are present in the majority of human ovarian carcinoma cells (244, 264-266). The ultimate goal of this study was to determine whether the regulation of GnRH-II by EGF is physiologically relevant to ovarian cancer cell invasiveness. Our initial experiment demonstrated that EGF induced GnRH-II mRNA levels in all three ovarian cancer cell lines (OVCAR-3, CaOV-3 and SKOV-3), and we wanted to know whether this increase of *GnRH-II* expression enhanced EGF-induced invasiveness in these cell lines. In support of this, we found that treatment with exogenous GnRH-II acts additively with EGF to promote the invasiveness of OVCAR-3 and CaOV-3 cells but not SKOV-3 cells. We also confirmed that neither GnRH-I or GnRH-II have any effect on SKOV-3 cell invasiveness (71), and we attributed this to the low GnRHR levels in SKOV-3 cells as compared with OVCAR-3 and CaOV-3 cells. Moreover, since the levels of GnRHR correlate with cancer grading and are elevated in advanced stage (stages III and IV) as compared to early stage (stages I and II) ovarian carcinomas (51), our findings support the clinical data and that GnRH-II promotes the EGF-induced invasiveness of ovarian cancer cells, and further corroborate the view that GnRH-II/GnRHR plays a crucial role in tumor progression/metastasis (71, 78).

The EGF receptor is expressed in 33–75% of ovarian tumors. It is also frequently amplified and/or over-expressed in ovarian cancer cells, when compared to normal ovarian surface epithelial cells, and transfection with an antisense construct of EGF receptor into human

ovarian cancer cell lines suppresses their malignant phenotype (132-134). Among the three ovarian cancer cell lines we tested, SKOV-3 cells expressed the most EGFR whereas OVCAR-3 cells expressed the least EGFR; this reflects in line with the fact that SKOV-3 cells have the highest basal invasiveness while OVCAR-3 is the least invasive cell line. In addition, exogenous GnRH-II treatment acts additively with EGF to promote the invasive properties of OVCAR-3 and CaOV-3 cells, and GnRHR appears to be essential for this effect because siRNA-mediated down-regulation of the GnRHR completely blocked it. The down-regulation of the endogenous GnRHR also partially reduced the EGF-induced invasion in OVCAR-3 and CaOV-3 cells and this supports our hypothesis that GnRH-II signaling is involved in the EGF-induced invasiveness of ovarian cancer cells. More importantly, EGF induced a transient increase in GnRHR levels in OVCAR-3 and CaOV-3 cells and this could serve to further enhance EGF/GnRH-II-induced invasion in these two cell lines. By contrast, EGF treatment did not affect the GnRHR levels in SKOV-3 cells; thus confirming our results that there is no additive effect by EGF and GnRH-II on SKOV-3 cells invasion.

In summary, our studies provide important insights into the molecular mechanism and physiological relevance of EGF-mediated *GnRH-II* expression in ovarian cancer. In this scenario (Fig. 3.7), we propose that EGF stimulation of ovarian cancer cells results in the autophosphorylation of the EGFR and induces ERK1/2 signaling, which subsequently enhances the phosphorylation of CREB and its binding with C/EBPβ. The dynamic tethering of p-CREB and C/EBPβ onto a CRE within the *GnRH-II* promoter then increases its transcriptional activity, and results in increased GnRH-II mRNA levels in ovarian cancer cells. This ultimately enhances their production and secretion of GnRH-II, which then participates in an autocrine/paracrine loop together with EGF to promote the invasiveness of ovarian cancer cells.
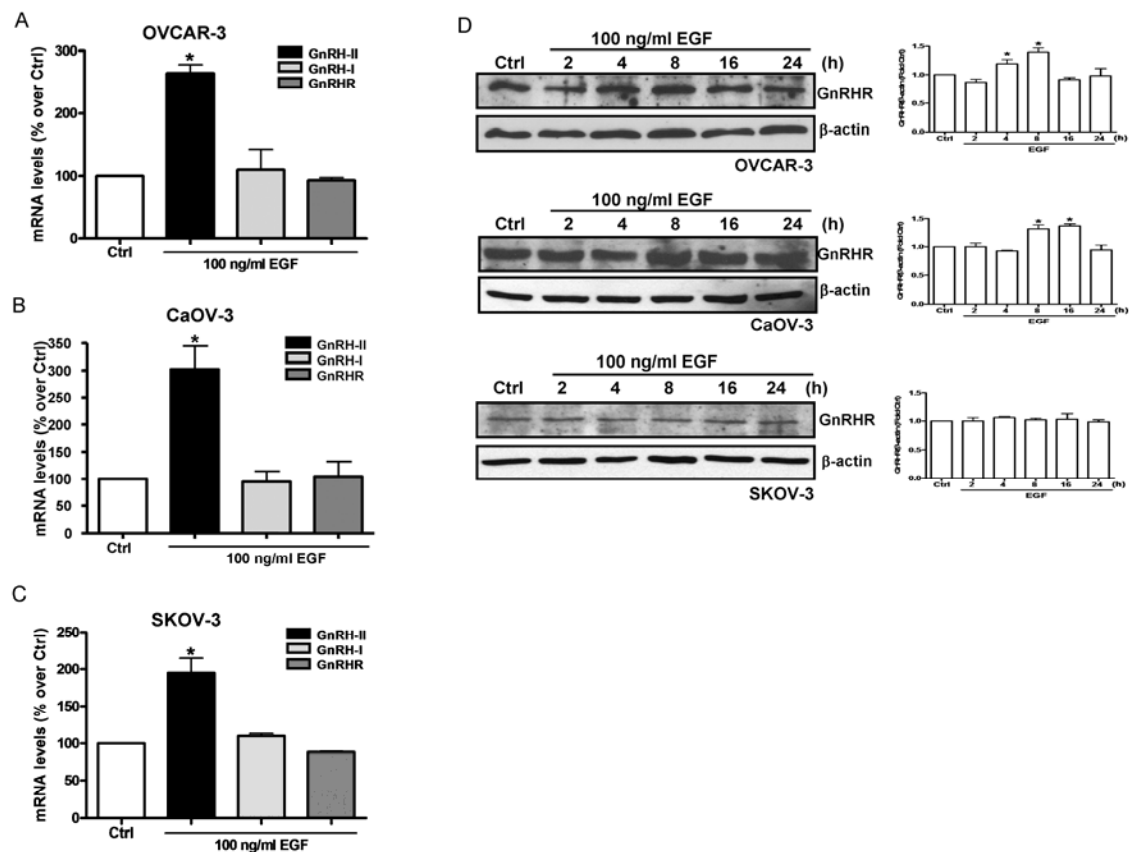
FIG. 3.1. EGF induces GnRH-II mRNA in ovarian cancer cell lines. (A) OVCAR-3 cells, (B) CaOV-3 cells and (C) SKOV-3 cells were treated with 100 ng/ml of EGF for 24 h. Total RNA was isolated and cDNA was used in real-time PCR to evaluate the effect of EGF on GnRH-II, GnRH-I and GnRHR mRNA levels expressed as percentage over control (Ctrl) level. * $p<0.05$ compared to untreated control (Ctrl). (D) OVCAR-3 cells, CaOV-3 cells and SKOV-3 cells were treated with 100 ng/ml EGF for increasing times (2, 4, 8, 16, 24 h). Cells harvested were then subjected to Western blotting and probed for GnRHR. β-actin was used as a normalization control. Left panels were representative Western blots. Right panels were quantitative results from three independent experiments. * $p<0.05$ compared to untreated control (Ctrl).
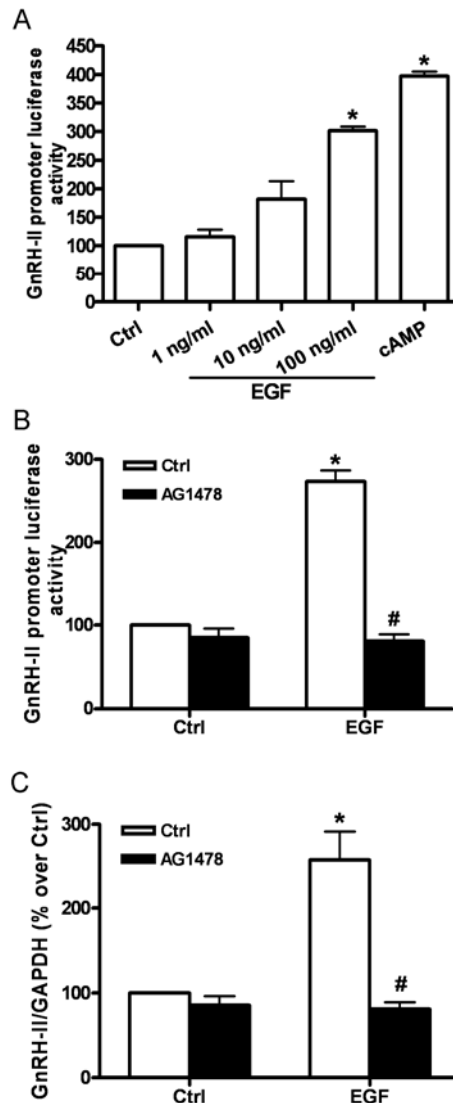
FIG. 3.2. EGFR-dependent activation is required for the stimulation of GnRH-II expression in OVCAR-3 cells. (A) OVCAR-3 cells were treated with increasing concentraion of EGF after transient transfection with a *GnRH-II* promoter-driven luciferase reporter gene construct together with a (RSV)-*lacZ* plasmid. (B) A similar experiment was performed in which 10 μM EGFR inhibitor, AG1478, was pre-treated for 30 min and then co-treated in the presence or absence of 100 ng/ml EGF in OVCAR-3 cells. Cell lysates were collected for luciferase assay and measurements of β-galactosidase activity as a control for transfection efficiency. Results are expressed as mean ± SEM luciferase activity/β-galactosidase activity (i.e., GnRH-II promoter luciferase activity) of three independent experiments. (C) In parallel experiments, total RNA was isolated after the administration of AG1478 in the presence or absence of 100 ng/ml EGF for 24 h and subjected for RT-qPCR to evaluate the effect of EGF on GnRH-II mRNA levels expressed as fold changes over control (Ctrl) levels in OVCAR-3 cells. * $p<0.05$ compared to untreated control (Ctrl). # $p<0.05$ compared to EGF treatment.
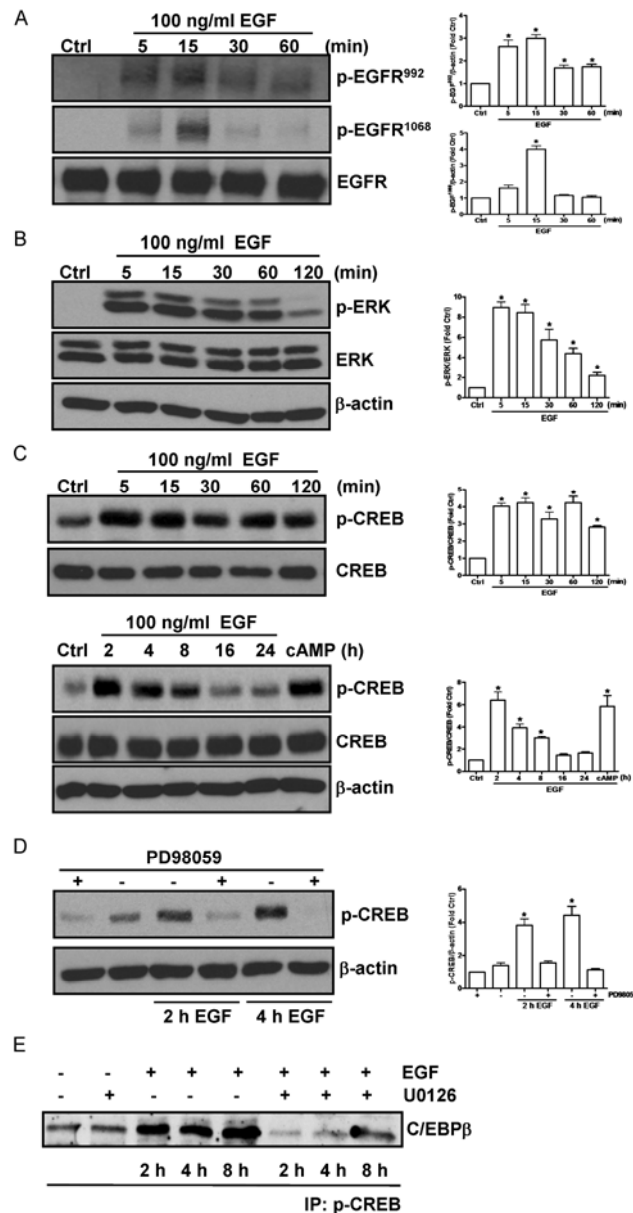
FIG. 3.3. EGF-activated ERK1/2 pathway is required for the phosphorylation of CREB and its interaction with C/EBPβ. (A) OVCAR-3 cells were treated with 100 ng/ml of EGF for different time slots (5, 15, 30, 60 min) or (2, 4, 8, 16, 24 h). Cells harvested were then subjected to Western blotting and probed for phosphorylated EGFR at different tyrosine (992 and 1045). (B, C) Nuclear lysates harvested from EGF treated cells were subjected to Western blotting and probed for phoshorylated ERK1/2 (p-ERK1/2) and phosphorylated CREB (p-CREB), respectively. (D) OVCAR-3 cells were pre-treated with 20 μM PD98059 (selective MAPK inhibitor) for 30 mins and then co-treated in the presence or absence of 100 ng/ml EGF for 2 h or 4 h. Nuclear cell lysates were collected and the level of p-CREB was determined by Western blotting. Total EGFR (EGFR), total CREB (CREB), total ERK1/2 (ERK1/2) and β-actin were used as normalization control. Left panels showed representative Western blots. Right panels were quantitative results from three independent experiments. * $p<0.05$ compared to untreated control (Ctrl).(E) OVCAR-3 cells were pre-treated with 20 μM U0126 (selective MAPK inhibitor) for 30 min and then treated in the presence or absence of 100 ng/ml EGF for 2, 4 or 8 h. Nuclear cell lysates were collected and subjected to immunoprecipitation (IP) with p-CREB antibody, and the IPs were Western blotted with C/EBPβ antibody. The Western blot is representative of IPs from three independent experiments.

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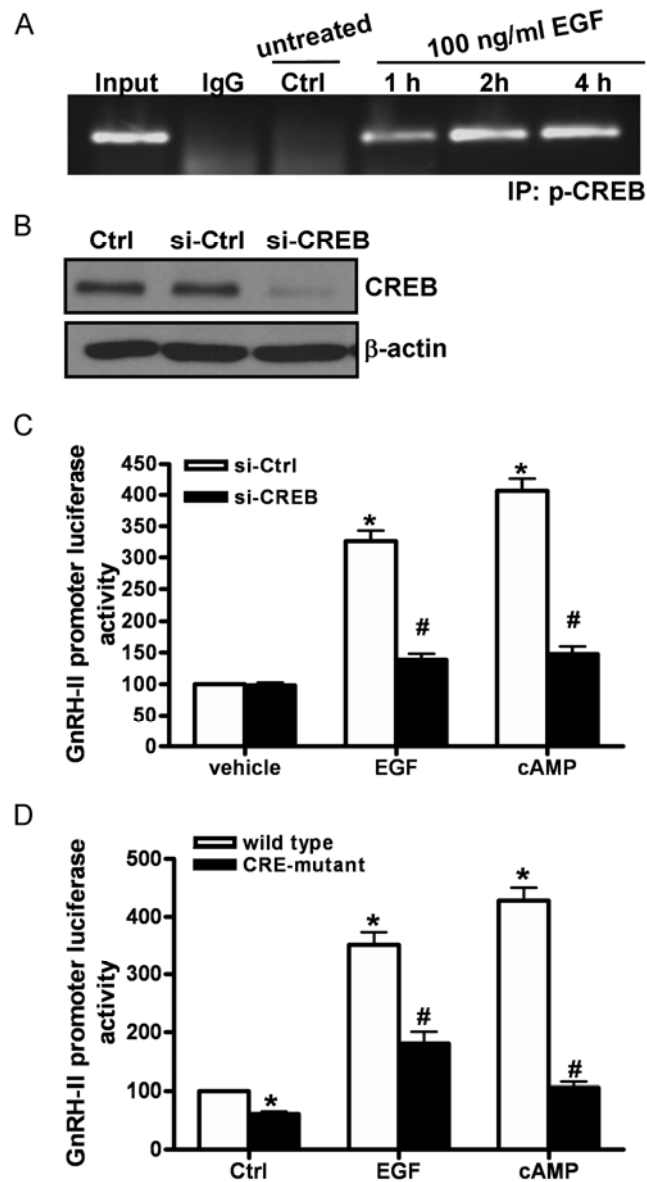
FIG. 3.4. EGF regulates the binding of p-CREB on CRE site in GnRH-II promoter. (A) OVCAR-3 cells were treated with 100 ng/ml EGF for 1 h, 2 h or 4 h, or were untreated (Ctrl). Cross-linked, sheared chromatin was immuno-precipitated (IP) with p-CREB and recovered chromatin was subjected to PCR analysis using primers spanning the CRE region of the *GnRH-II* promoter. The IgG lanes are ChIPs performed using non-specific IgG. An ethidium bromide-stained gel of PCR products shows a representative of ChIP analysis from three independent experiments. (B) The efficiency of the siRNA was tested by Western blotting for CREB (67.5% knockdown) in OVCAR-3 cells. (C) Cells were transfected with *GnRH-II* promoter-driven luciferase reporter gene construct together with 150 nM random siRNA controls (si-Ctrl) or siRNAs for CREB respectively, and then treated with 100 ng/ml EGF for 24 h. (D) OVCAR-3 cells were transfected with wild type *GnRH-II* promoter-driven lucifease reporter gene construct or a 3 bp mutated CRE-*GnRH-II* promoter-driven luciferase reporter construct followed by 100 ng/ml EGF or 1 mM cAMP treatment. Cell lysates were assayed for luciferase activity and measurements of β-galactosidase activity as a control for transfection efficiency, the result of which are expressed as mean ± SEM luciferase activity/β-galactosidase activity (i.e., GnRH-II promoter luciferase activity) of three independent experiments.* $p<0.05$ compared to cells treated with a siRNA control (si-Ctrl). # $p<0.05$ compared to cells treated with specific CREB siRNA and followed by EGF treatment.

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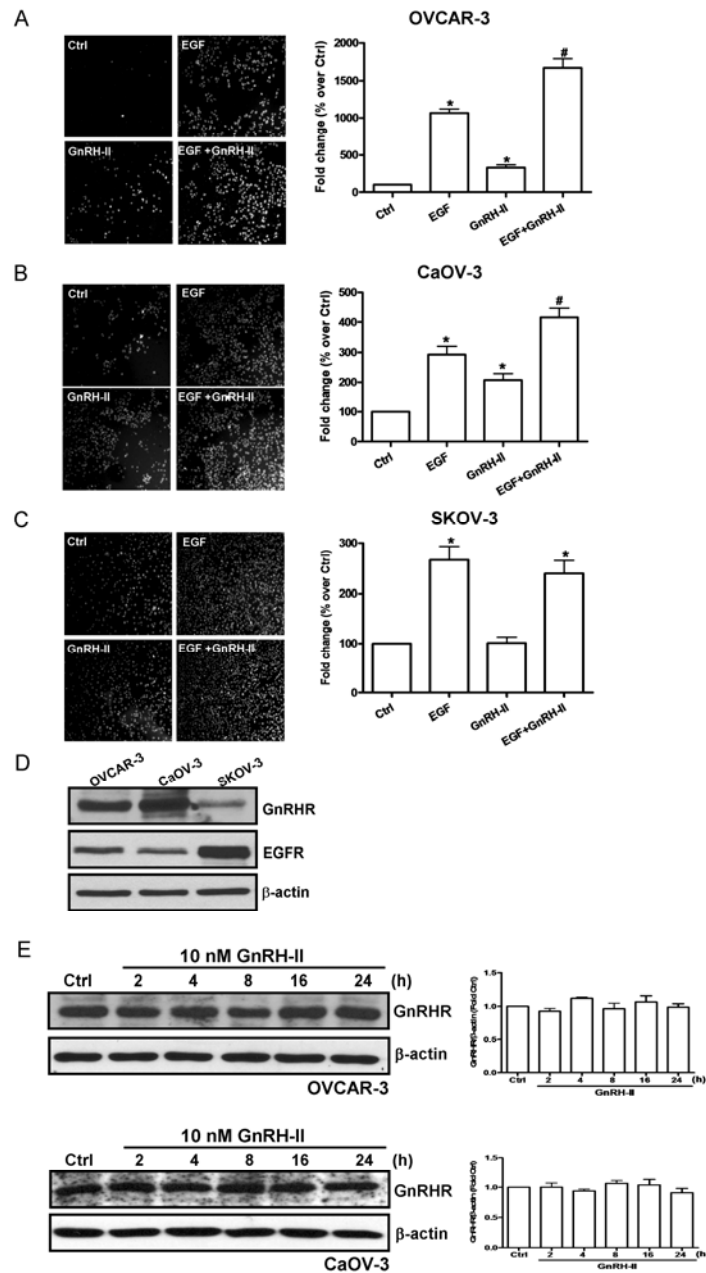
FIG. 3.5. GnRH-II acts additively with EGF to promote ovarian cancer cell invasion. (A) OVCAR-3 cells (B) CaOV-3 cells and (C) SKOV-3 cells were treated with 100 ng/ml of EGF, 10 nM of GnRH-II or in combination for 24 h and then seeded into Matrigel-coated transwells and cultured for 48 h. Non-invading cells were wiped from the upper side of the filter and nuclei of invading cells were stained with Hoechst 33258. Left panel shows representative photos of invasion assay; right panel shows summarized quantitative results. Results are expressed as mean ± SEM of at least three independent experiments. *$p<0.05$ compared with untreated control (Ctrl). #$p<0.05$ compared with EGF or GnRH-II treatment. (D) The endogenous expression of GnRHR and EGFR in OVCAR-3, CaOV-3 and SKOV-3 cells were examined by Western blotting. E, OVCAR-3 cells and CaOV-3 cells were treated with 10 nM of GnRH-II for 2, 4, 8, 16 or 24 h. Cells were harvested and protein extracts were subjected to Western blotting and probed for GnRHR or β-actin as a normalization control. Left panels showed representative Western blots. Right panels showed quantitative results from three independent experiments.
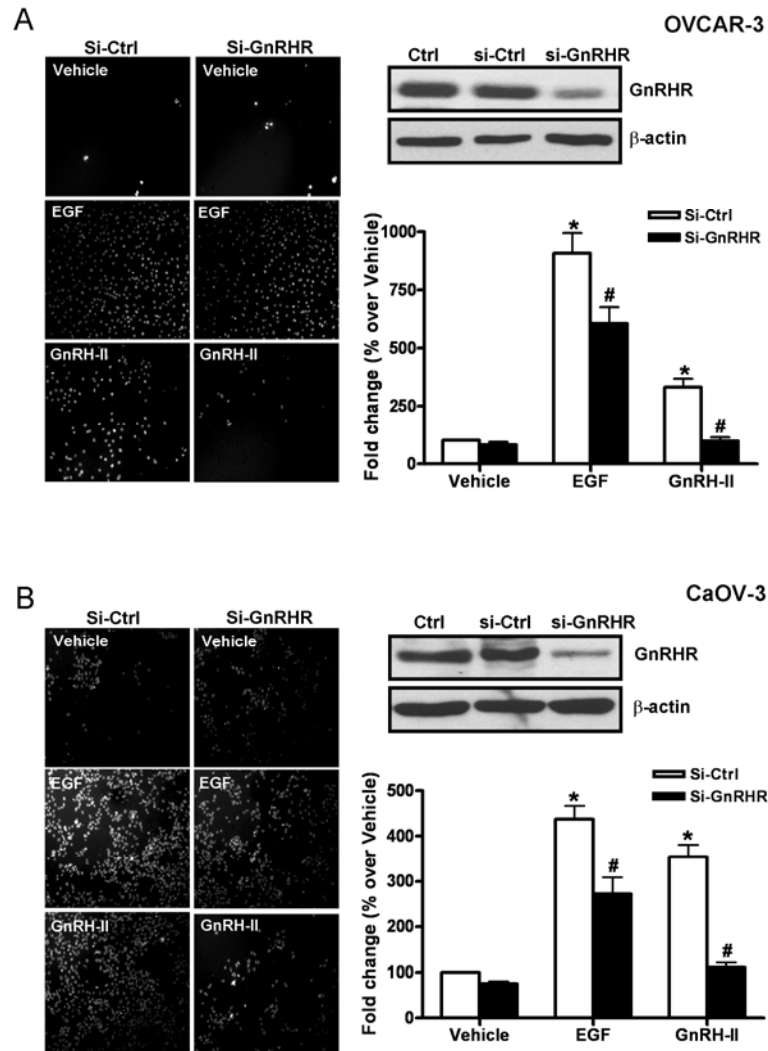
FIG. 3.6. GnRH-II signaling is involved in EGF-induced ovarian cancer cell invasion. (A) OVCAR-3 cells and (B) CaOV-3 cells were transfected with 100 nM type I GnRH receptor siRNA (si-GnRHR) or 100 nM siRNA controls (si-Ctrl) respectively, and followed by 100 ng/ml EGF or 10 nM GnRH-II treatment. Treated cells were then seeded into Matrigel coated transwells and cultured for 48 h. Non-invading cells were wiped from the upper side of the filter and nuclei of invading cells were stained with Hoechst 33258. Left panel shows representative photos of the invasion assay, right panel shows summarized quantitative results. Results are expressed as mean ± SEM of at least three independent experiments. *p<0.05 compared with untreated control (Ctrl). [#]p<0.05 compared with EGF or GnRH-II treatment. The efficiency for GnRHR siRNA was tested by Western blotting.
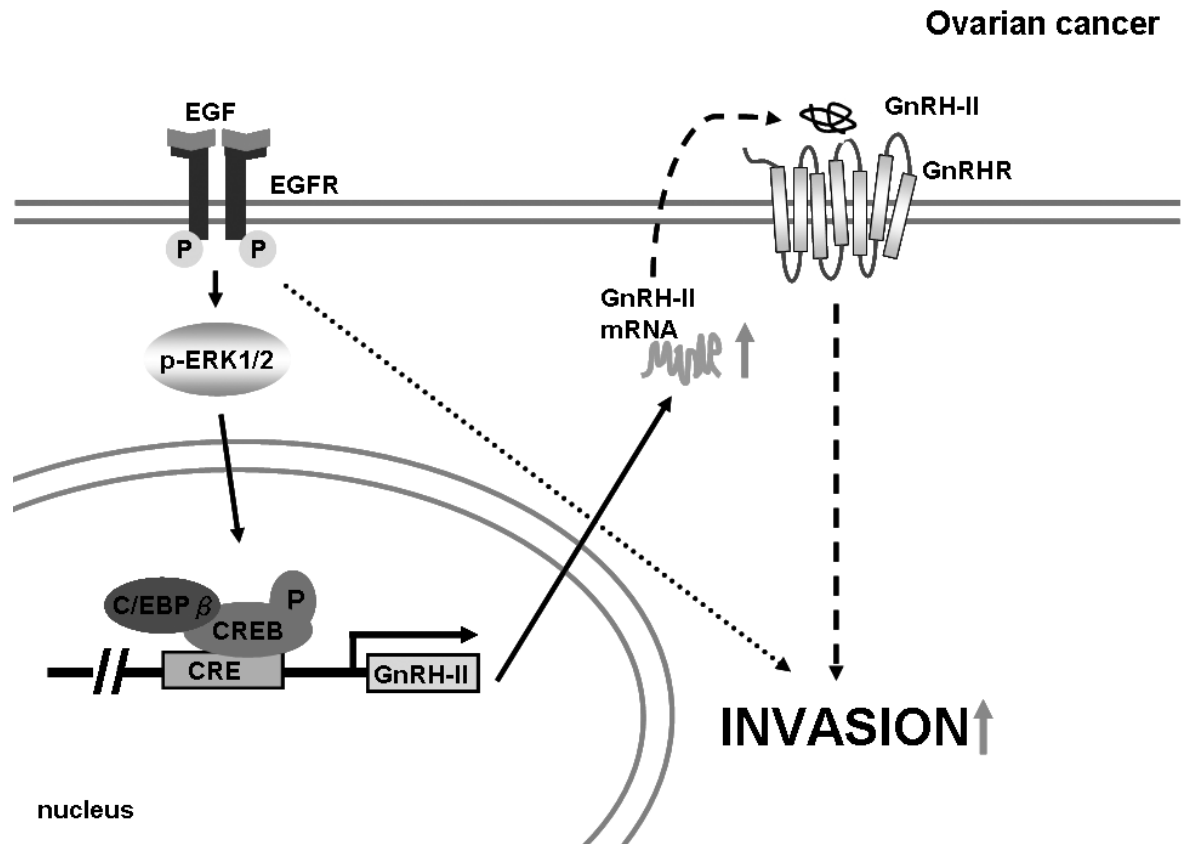
FIG. 3.7. Proposed model for EGF induced GnRH-II synthesis contributes to ovarian cancer cell invasion. EGF acting on its receptor induces the autophosphorylation of EGFR thereby stimulating the phosphorylation of ERK1/2. Phosphorylated ERK1/2 then translocates into the nucleus and mediates the phosphorylation of CREB, the recruitment of C/EBPβ and the binding of p-CREB onto the CRE site of the GnRH-II promoter. Up-regulated *GnRH-II* transcription then increases GnRH-II synthesis. Increased production of GnRH-II acts in an autocrine manner through the GnRHR, the levels of which may also be increased by EGF treatment, to stimulate the invasive potential of ovarian cancer cells. EGFR, EGF receptor; GnRHR, GnRH receptor; p-ERK1/2, phosphorylated ERK1/2; p-CREB, phosphorylated CREB; CCAAT/enhancer binding protein beta, C/EBPβ, CRE, cAMP responsive element.

CHAPTER 4. 37 kDa Laminin receptor precursor mediates gonadotropin-releasing hormone-II- induced matrix metalloproteinase-2 expression and invasiveness in ovarian cancer cells

4.1 Introduction

It is increasingly apparent that gonadotropin-releasing hormone-II (GnRH-II) acts as an autocrine regulator in non-pituitary tissues in addition to its role in promoting gonadotropin synthesis and steroid hormone production (52, 53), and GnRH-II is emerging as an important player in cancer cell biology (71). We have previously reported that epidermal growth factor increases GnRH-II production in ovarian cancer cells, and that this enhances their invasive potential by binding to the GnRH type I receptor (GnRHR) (53), but the downstream mechanisms responsible for this autocrine action of GnRH-II remained to be defined.

Matrix metalloproteineases (MMPs) are a family of secreted or membrane-bound enzymes that degrade extracellular matrix (ECM) molecules, thereby facilitating cancer cell migration or invasion (267). The expression of MMP-2 and MMP-9 is significantly higher in ovarian cancer, as compared with normal ovarian tissues (144, 150, 166). Moreover, *in vitro* studies have demonstrated that MMP-2 (268) and MMP-9 (269, 270) contribute to the invasive and metastatic potential of ovarian carcinoma, and that GnRH-I acts through the GnRHR to increase their proteolytic activities in ovarian cancer cell lines (78). Interestingly, the 37 kDa precursor (LRP) has also been reported to increase the activity of MMP-2, and thereby promote breast cancer cell (202) and melanoma cell (271) invasiveness.

The LRP homodimerizes to form the 67 kDa non-integrin laminin receptor (67LR) (178, 272), and an increase in 67LR levels has been found in a variety of common cancers, as compared with their corresponding normal tissues (177, 273). In many cases, a positive correlation between LRP expression and tumor aggressiveness or metastatic potential has also been found (177). At the cell surface, 67LR can interact with laminin and this is thought to

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influence tumor cell-attachment (198, 274, 275), migration (276), angiogenesis (277), invasion and metastasis (198, 274, 278). A palindromic sequence (LWMMWL) within LRP acts as the major laminin-1 binding site of 67LR (182, 187, 188, 194), and a synthetic polypeptide (peptide G) that includes this sequence binds to laminin-1 with high affinity and can thereby block the interaction between the 67LR and laminin-1 (187). In addition, the amount of 67LR on the cell surface is increased by GnRH-I and GnRH-II in normal and cancerous T cells, and this induces their invasive and metastatic potential (20). We have therefore explored the possibility that LRP influences the way that GnRH-II acts in an autocrine manner to increase ovarian cancer invasion *via* altered MMP expression.

4.2 Materials and methods

Cells and cell culture

The human ovarian adenocarcinoma cell lines, OVCAR-3, CaOV-3 and SKOV-3 were obtained from American Type Culture Collection (Manassas, VA, USA). Normal ovarian surface epithelial cells were obtained by scraping the ovarian surface at surgery or laparoscopy for non-malignant disorders, as previously described (279). Cell cultures were maintained in M199/MCDB105 (Invitrogen Inc., Burlington, ON, Canada) supplemented with 10% fetal bovine serum (FBS; Hyclone Laborataries Inc., Logan, UT, USA) at 37 C in a humidified atmosphere of 5% CO_2. The cells were sub-cultured when they reached about 90% confluence using a trypsin/EDTA solution (0.05% trypsin, 0.5 mM EDTA).

Antibodies and reagents

GnRH-II analog (DArg6-Azagly10-GnRH-II) was purchased from Bachem (Belmont, CA). Peptide G (IPCNNKGAHSVGLMWWMLAR), corresponding to amino acids 161-180 of LRP was synthesized at the University of British Columbia (Vancouver, BC, Canada). The

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monoclonal LRP antibody (MLuC5) and the polyclonal β-actin antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The monoclonal GnRHR antibody, monoconolal MMP-2 antibody and monoclonal MMP-9 antibody were obtained from Neomarkers (Fremont, CA). Horseradish peroxidase-conjugated goat anti-rabbit IgG and goat anti-mouse IgG were obtained from Bio-Rad Laboratories (Hercules, CA). Horseradish peroxidase-conjugated donkey anti-goat IgG was obtained from Santa Cruz Biotechnology.

Reverse transcription quantitative real-time PCR (RT-qPCR)

After treatment with 10 nM GnRH-II, medium was removed from the culture dish and RNA was extracted using Trizol (Invitrogen). Total RNA (2 μg) was reverse transcribed into cDNA using a first-strand cDNA synthesis kit (GE Healthcare Bio-Science, Piscataway NJ, USA) following the manufacturer's procedure. The primers used for SYBR Green RT-qPCR were designed using Primer Express Software v2.0 (Applied Biosystems, Foster City, CA, USA). The primers for GnRH-II mRNA are: sense, 5′-CTGCTGACTGCCCACCTT; and antisense, 5′-GCTTTCCTCCAGGGTACCAG. The primers for GnRHR are: sense, 5′-ACTGTTCCGACTTTGCTGTTGCT; and antisense, 5′-ACCGCTCCCTGGCTATCAC. The primers for LRP are: sense, 5'- ATGTCCTGCAAATGAAGGAGG; and antisense, 5'-TGGAAGTCAAGATTGGTGCCA. The primers for MMP-2 are: sense, 5'-TACACCAAGAACTTCCGTCTGT; and antisense, 5'-AATGTCAGGAGAGCTCCCCATA. The primers for MMP-9 are: sense, 5'-GCCACTACTGTGCCTTTGAGTC; and antisense, 5'-CCCTCAGAGAATCGCCAGTACT. The primers for GAPDH are: sense, 5'-GAGTCAACGGATTTGGTCGT; and antisense, 5'-GACAAGCTTCCCGTTCTCAG. The reactions were performed using SYBR Green PCR Master Mix (Applied Biosystems). All RT-qPCR experiments were run in triplicate and a mean value was used for the determination of mRNA levels. Negative controls containing water instead of sample cDNA were used in each

experiment. Relative quantification of the mRNA levels of GnRH-II, GnRHR, LRP, MMP-2 and MMP-9 was performed using the comparative Cq method with GAPDH as the reference gene and with the formula $2^{-\Delta\Delta Cq}$.

Plasmid constructs and cell transfections

The LRP and eGFP cDNA constructs in the same plasmid expression vector (pReciever-M02) were purchased from GeneCopoeia (Rockville, MD). The LMWWML site within coding sequence of the LRP expression plasmid was mutated to the corresponding sequence of the 37 kDa LRP orthologs found in *Arabidopsis* (CLFWLL) and *Saccharomyces* (LIWYLL) because they lack laminin-binding activity but retain other highly-conserved functions of LRP (181). Mutagenesis was performed using the Quickchange II XL site directed mutagenesis kit (Stratagene, La Jolla, CA, USA), with the LRP construct as template and the following oligonucleotide primers: LRP Mut A (mutated sequence underlined encodes CLFWLL): forward, 5'-CTCAGTGGG<u>TTGCTTGTTCTGGTT</u>GCTGGCTCGGGAAGTTCTG and reverse, 5'- CAGAACTTCCCGAGCCAGC<u>AACCAGAACAAGCAA</u>CCCACTGAG; LRP Mut B, (mutated sequence underlined encodes LIWYLL): forward, 5'-CTCAGTGGGTTT<u>GATCTGGTACTT</u>GCTGGCTCGGGAAGTTCTG and reverse, 5'-CAGAACTTCCCGAGCCAGC<u>AAGTACCAGATC</u>AAACCCACTGAG. The mutated constructs were sequenced to verify that only the desired mutations had occurred.

CaOV-3 cells over-expressing eGFP, wild-type LRP (LRP), or mutated LRP (Mut A and Mut B) were selected with 600 μg/ml geneticin (Invitrogen) and cloned by limiting dilution. The eGFP expression vector was used as a transfection control, and clones expressing eGFP were verified by fluorescence microscopy, while LRP mRNA and protein levels in clones expressing wild-type or mutant LRP were measured by RT-qPCR or Western blotting.

Transfections of expression plasmids were performed using Lipofectamine LTX and PLUS Reagent (Invitrogen), while siRNA transfections were carried out using Lipofectamine RNAiMAX Reagent (Invitrogen) following the manufacturer's protocol. Briefly, 5×10^5 cells were seeded into six-well tissue culture plates one day prior to transfection with 100 nM GnRH-II siRNA (si-GnRH-II), 100 nM GnRHR siRNA (si-GnRHR), 75 nM 37kDa LRP siRNA (si-LRP) or a non-targeting control siRNA (si-Ctrl) (Dharmacon, Inc., Lafayette CO). After 6 h, the medium in each well was replaced with 2 ml of 0.5% FBS medium and the cells were further incubated overnight (18 h). The culture medium was then removed and the cells were treated with 10 nM GnRH-II in 0.5% serum medium for the times indicated.

Adhesion assay

Equal amounts of laminin (2 μg/cm^2), fibronectin (5 μg/cm^2) and growth-factor reduced Matrigel (5 μg/cm^2) were used to coat 96-well tissue culture plates (Becton Dickinson Labware, Franklin Lakes, NJ) for 2 h at 37 C. Cells were trypsinized and seeded in the coated tissue culture plates in serum free medium and allowed to adhere at 37 C for 8 h. The plates were then washed twice with cold PBS and the adherent cells were fixed with ice-cold methanol, stained with crystal violet and quantified by measuring absorbance at 630 nm.

Invasion assay

Transwell cell culture inserts (8 μm pore size, 24-well, BD Biosciences) were coated with 40 μl of 1 mg/ml growth-factor reduced Matrigel (BD Biosciences). Cells in M199/MCDB105 medium supplemented with 0.5% FBS were incubated for 48 h against a gradient of 10% FBS for OVCAR-3 cells (1.5×10^6/insert) and CaOV-3 cells (1.25×10^6/insert) and 24 h of 5% FBS for SKOV-3 cells (1×10^6 /insert). Cells on the lower side of the

membrane were fixed with ice-cold methanol, stained with Hoechst 33258 (Sigma), and the number of nuclei were counted using a Zeiss Axiophot epifluorescent microscope equipped with a digital camera and Northern Eclipse 6.0 software (Empix Imaging, Mississauga, ON). Individual experiments had duplicate inserts and five microscopic fields were counted per insert.

Data analysis

Results are shown as the mean ± SEM of three independent experiments and were analyzed by one-way ANOVA followed by Tukey test using PRISM software (GraphPad Software Inc., San Diego, CA, USA). Means were considered significantly different from each other at $P < 0.05$.

4.3 Results

GnRH-II modulates ovarian cancer cell invasion in an autocrine manner

Treatment of ovarian cancer cells with epidermal growth factor increases their GnRH-II mRNA levels and this contributes to their invasive potential (53). We have now treated two ovarian cancer cell lines with GnRH-II siRNA prior to an invasion assay, and this demonstrated that knockdown of GnRH-II mRNA levels in OVCAR-3 (Fig. 4.1A) and CaOV-3 (Fig. 4.1B) cells significantly reduces their invasive properties (Fig. 4.1C and D). Importantly, this GnRH-II siRNA-mediated reduction in the invasiveness of both cell lines was reversed upon exogenous treatment of GnRH-II (Fig. 4.1C and D). These results confirm and extend our previous finding that GnRH-II functions in an autocrine manner to modulate the invasive potential of ovarian cancer cells.

GnRH-II increases LRP production in ovarian cancer cells

Over-expression of LRP influences cancer cell metastasis and invasiveness (177), and we therefore compared the LRP mRNA levels in normal ovarian surface epithelial cells and three serous ovarian carcinoma cell lines (i.e. OVCAR-3, CaOV-3 and SKOV-3 cells). The results indicate that LRP mRNA levels are much higher in CaOV-3 and SKOV-3 cells than in OVCAR-3 cells or normal ovarian surface epithelial cells (Fig. 4.2A, upper panel). These three ovarian cancer cell lines exhibit different invasive properties, and this is evident in our invasion assays. For instance, SKOV-3 cells were seeded at the lowest cell density but demonstrate the highest cell invasiveness, while CaOV-3 and OVCAR-3 cells were seeded at a higher cell density and show a lower cell invasive potential (Fig. 4.2A, lower panel). Interestingly, the LRP mRNA levels in these three ovarian cancer cell lines corresponds to their ranking in terms of invasiveness (SKOV-3> CaOV-3> OVCAR-3) (Fig. 4.2A).

To examine whether GnRH-II treatment induced LRP production in ovarian cancer cells, OVCAR-3 and CaOV-3 cells were treated with 10 nM GnRH-II for 8 h or 24 h prior to harvesting for measurements of LRP expression. As shown in Figure 4.2B, GnRH-II increased LRP mRNA and LRP levels in both OVCAR-3 and CaOV-3 cells, which express relatively high levels of GnRHR; whereas SKOV-3 cells, a cell line that expresses limited amounts of GnRHR (53), did not respond in this way to GnRH-II treatment (Fig. 4.2B). This is in accordance to our previous finding that GnRH-II treatment does not affect the invasive potential of SKOV-3 cells (53). Furthermore, when SKOV-3 cells were treated with GnRHR siRNA prior to invasion assays, the results show that depletion of GnRHR in this cell line does not have any effects on its basal invasiveness (Fig. 4.3).

A GnRH-II/GnRHR autocrine loop regulates LRP levels in ovarian cancer cells

When OVCAR-3 (Fig. 4.2C) and CaOV-3 cells (Fig. 4.2D) were treated with GnRH-II siRNA, a reduction in LRP levels was observed that could be reversed by treatment of the cells with exogenous GnRH-II. To verify that GnRH-II acts through the GnRHR to regulate LRP production, the cells were treated with GnRHR siRNA prior to treatment with GnRH-II. The results show that depletion of GnRHR in OVCAR-3 (Fig. 4.2E) and CaOV-3 cells (Fig. 4.2F) inhibits both basal and GnRH-II-induced LRP levels, without altering GnRH-II mRNA levels (Fig. 4.4). These data suggest that GnRH-II acts *via* the GnRHR in an autocrine manner to regulate LRP production in ovarian cancer cells.

LRP promotes ovarian cancer cell invasion

To assess the role of LRP in ovarian cancer cell invasion, siRNA was used to knockdown LRP levels in CaOV-3 (Fig. 4.5A) and SKOV-3 cells (Fig. 4.5B) prior to invasion assays using Matrigel-coated transwells. Cells treated with LRP siRNA exhibited a significant reduction in their invasiveness, as compared with cells treated with control siRNA, suggesting that LRP up-regulates this property of ovarian cancer cells.

Peptide G mimics the laminin binding site of LRP, and will competitively block interactions between cell surface 67LR and laminin within an ECM (204). Laminin is a major component of the synthetic ECM, Matrigel, and the basal invasiveness of CaOV-3 (Fig. 4.5C) and SKOV-3 cells (Fig. 4.5D) was reduced by ~50% when peptide G was pre-incubated with Matrigel-coated transwells. We therefore also conducted adhesion assays to determine whether the effects of LRP on ovarian cancer cell invasion are simply caused by a loss of cell adhesion. In laminin-coated tissue culture plates, depletion of LRP with siRNA treatment in CaOV-3 cells resulted in an ~75% inhibition of cell adhesiveness; while only an ~20% inhibition of cell adhesion was observed in Matrigel-coated tissue culture plates (Fig. 4.5E). In addition, pre-

incubation of laminin-coated tissue culture plates with peptide G reduced the adhesion of CaOV-3 cells by ~60%, while peptide G treatment of Matrigel-coated plates reduced the adhesiveness of these cells to the ECM by only ~15% (Fig. 4.5F). Thus, while these results suggest that the interaction between 67LR and laminin facilitates CaOV-3 cell adhesion to the ECM, it does not entirely account for the adhesive properties of ovarian cancer cells or the substantial loss in their invasiveness when LRP levels were depleted.

To further verify that LRP promotes ovarian cancer cell invasion, we first stably transfected CaOV-3 cells with a LRP expression vector, and identified two independent CaOV-3 cell clones that over-express LRP by Western blotting (Fig. 4.6A). Using transwell invasion assays, we found that the LRP over-expressing CaOV-3 cells were significantly more invasive than CaOV-3 cells stably transfected with a control expression vector (Fig. 4.6B). Furthermore, this increased invasiveness of LRP over-expressing cells was abolished when they were pre-treated with LRP siRNA (Fig. 4.6C). Moreover, when CaOV-3 cells were engineered to over-express a laminin-binding deficient LRP, they showed no change in invasiveness (Fig. 4.7A) or adhesion to Matrigel (Fig. 4.7B). Taken together, these results suggest that increases in LRP production enhance ovarian cancer cell invasiveness *in vitro* through an increase in the amounts of cell surface 67LR that can interact with laminin within Matrigel.

LRP is an obligate intermediate in GnRH-II-induced MMP-2 production and ovarian cancer cell invasion

Since it has been reported that LRP modulates the expression of MMP-2 in breast cancer cells (202), we examined whether altered LRP levels influences MMP-2 or MMP-9 expression in OVCAR-3 and CaOV-3 cells (Fig. 4.8). In these experiments, siRNA-mediated knockdown of LRP levels decreased the basal mRNA levels of MMP-2 but not MMP-9 in both cell lines (Fig. 4.8A, C, D and F). Moreover, transient over-expression of LRP in these cells specifically

increased the levels of MMP-2 mRNA, but not MMP-9 mRNA (Fig. 4.8B, C, E and F). In addition, no increases in MMP-2 mRNA (Fig. 4.8G) or MMP-2 (Fig. 4.8H) levels were detected in CaOV-3 cells over-expressing the laminin-binding deficient LRP mutant. These experiments indicate that an interaction between the 67LR and laminin specifically increases MMP-2 production in ovarian cancer cells.

In line with a previous report that GnRH-I/GnRHR signaling up-regulates *MMP-2* expression in ovarian cancer cells (78), we also found that treatment of OVCAR-3 (Fig. 4.9A) and CaOV-3 cells (Fig. 4.9B) with GnRH-II increased MMP-2 mRNA levels in a time-dependent manner. In addition, depletion of GnRHR in OVCAR-3 and CaOV-3 cells abolished the GnRH-II-stimulated increases in MMP-2 levels (Fig. 4.9C and D) in both cell lines, and confirmed that GnRH-II/GnRHR interactions stimulate MMP-2 expression. In addition, we further verified that GnRH-II treatment did not affect MMP-2 mRNA levels in SKOV-3 cells (Fig. 4.10A), which are known to have very low GnRHR levels (53). Moreover, depletion of GnRHR levels in SKOV-3 cells by GnRHR siRNA treatment does not influence MMP-2 levels in these cells (Fig. 4.10B). Importantly, depletion of LRP by LRP siRNA treatment abolished the GnRH-II-induced increase in MMP-2 production in OVCAR-3 (Fig. 4.9E) CaOV-3 cells (Fig. 4.9F).

To explore this further, CaOV-3 cells were treated with LRP siRNA or MMP-2 siRNA prior to invasion assays, and the results of these experiments indicate that depletion of either LRP (Fig. 4.11A) or MMP-2 (Fig. 4.11C) abolishes GnRH-II-induced invasion. Furthermore, depletion of MMP-2 levels in CaOV-3 cells did not affect GnRH-II, GnRHR or LRP levels in this cell line (Fig. 4.12). Importantly, pre-incubation of Matrigel-coated transwells with peptide G attenuated the pro-invasive effects of GnRH-II in CaOV-3 cells (Fig. 4.11B). These observations suggest that LRP is a key intermediary in GnRH-II-stimulated MMP-2 production and may thereby play a pivotal role in GnRH-II-induced ovarian cancer cell invasiveness.

4.4 Discussion

Both GnRH-II (139) and GnRHR (280) are more abundant in malignant ovarian tumors than in benign ovarian tissues, and this underscores the importance of understanding GnRH-II function in the context of ovarian cancer. Building on evidence that GnRH-II acts in an autocrine manner to promote ovarian cancer cell invasion (53), we have now found that this action of GnRH-II involves a GnRHR-dependent up-regulation of LRP production. This was not completely surprising because LRP has been reported to be a downstream target of GnRH-II/GnRHR signaling in T cell lymphomas, and to be associated with the metastatic potential of these cancer cells (20). However, our results also show that LRP is a critical intermediary in GnRH-II actions that lead to an increase in the invasive properties of OVCAR-3 and CaOV-3 cells. We believe this is important because LRP can dimerize and form the cell surface 67LR that functions as a binding protein for laminin in the ECM (177). In support of this, we used peptide G to block cell surface 67LR interactions with laminin during our ovarian cancer cell adhesion and invasion assays, and we found that this reduces the GnRH-II enhanced invasive potential of OVCAR-3 and CaOV-3 cells. This is important because increased 67LR levels have been observed in a wide range of malignancies, including ovarian carcinomas (205, 206), and correlate with minimal differentiation, disease progression and poor survival (180, 281). Accordingly, the invasive potential of the ovarian cancer cell lines we have used in our studies is directly related to their LRP levels, and this behavior can be manipulated by changing their LRP levels.

Interactions between cancer cells and laminin in the ECM promote tumor dissemination by several mechanisms, including increasing cell proliferation, adhesion, migration or invasion (282-284). In our *in vitro* studies, altered LRP levels did not influence ovarian cancer cell proliferation (data not shown). However, while the adhesion of these cells to laminin-coated tissue culture plates was largely dependent on the expression of LRP, their adhesion to an

artificial ECM (Matrigel) was only modestly affected by altering LRP expression. This suggested to us that the requirement for LRP, in relation to the GnRH-II induced invasive properties of ovarian cancer cells, can not simply be attributed to an alteration in adhesion to the ECM.

Although it is known that LRP is a multi-functional protein in eukaryotic cells and that 67LR also acts as an endocytotic receptor (177), our data imply that the laminin-binding domain of LRP plays a key role in the invasion of ovarian cancer cells after treatment of GnRH-II. The importance of interactions between cancer cells and ECM components, like laminin, is well documented in terms of their metastatic potential (285-288). It is also known that cancer cells interact with laminin *via* several cell-surface laminin-binding proteins, including dystroglycan and the integrins α1β1, α2β1, α3β1, α6β1, α6β4 and α7β1, as well as 67LR (201). Our data indicate that LRP plays a key role in GnRH-II induced increases in the invasive properties of ovarian cancer cells, and that this effect most likely relies on an interaction between the cell surface 67LR and laminin. Although the 67LR is not thought to act independently as a cell surface signaling molecule (177), it may act as an accessory with other cell surface laminin-binding proteins that function as *bona fide* signaling molecules upon interaction with laminin at sites other than that recognized by 67LR (177). For example, 67LR physically associates with α6β4 integrin (200) and co-regulation of their membrane localization is important for tumorigenesis (180, 281). In addition, up-regulation of α6β4 integrin increases its interaction with laminin and activates mitogen-activated protein kinase signaling (3), and also appears to stimulate the production of proteases, including MMP-2, MMP-9 and MT1-MMP, that are important in ECM degradation during cell invasion (289).

Laminin induces the production of proteases, like MMPs, as one mode of promoting cancer cell invasion (290). It has previously been reported that GnRH-I treatment of ovarian

cancer cells increases MMP-2 and MMP-9 production (78), and we have now shown that the autocrine actions of GnRH-II act through the GnRHR to exert the same effects. Others have shown that siRNA-mediated depletion of 67LR levels significantly reduces tumor aggressiveness with a decrease in the MMP-2 mRNA levels and proteolytic activity of melanoma cells (271). Likewise, we have found that LRP levels influence MMP-2 but not MMP-9 expression in ovarian cancer cells. Importantly, this effect was not seen when a laminin-binding deficient LRP was over-expressed in these cells, suggesting that the specific induction of MMP-2 expression is entirely dependent on the laminin-binding properties of LRP or the 67LR. Thus, our data also imply that the GnRH-mediated induction of MMP-9 expression in ovarian cancer cells must be mediated by some other pathway unrelated to the increased expression of LRP. However, we also conclude that LRP-induced *MMP-2* expression must be the predominant mechanism responsible for the autocrine actions of GnRH-II in relation to its effects on the increased invasive potential of some ovarian cancer cells. It should also be noted that MMP-2 is synthesized as a latent zymogen that requires proteolytic cleavage by membrane type I matrix metalloproteinase (MT1-MMP) to achieve its full proteolytic potential (155, 291). This is of interest because we have also recently obtained evidence that MT1-MMP is a downstream target of GnRH-II/GnRHR signaling (Poon SL *et al*., unpublished), and is independent of the actions of LRP (data not shown).

We and others have demonstrated previously that GnRH-I and GnRH-II have no effect on SKOV-3 cell invasiveness, and we attributed this to the low GnRHR levels in SKOV-3 cells as compared with OVCAR-3 and CaOV-3 cells (53). Our results also now indicate that GnRH-II treatment does not affect LRP or MMP-2 levels in SKOV-3 cells. In addition, GnRHR siRNA treatment has no effects on SKOV-3 cell invasiveness or LRP and MMP-2 levels. However, siRNA-mediated reductions in LRP levels or peptide G treatment markedly reduce the invasive potential of SKOV-3 cells. Thus, while LRP contributes to the invasive properties of SKOV-3

cells, this is not influenced by the GnRH-II/GnRHR autocrine loop as in OVCAR-3 and CaOV-3 cells, and other mechanisms may influence LRP expression in SKOV-3 cells.

Our present studies provide important insights into a novel aspect of GnRH-II function in ovarian cancer cell biology. In summary, we propose that the epidermal growth factor-stimulated increase in GnRH-II production by ovarian cancer cells (53) subsequently acts in an autocrine manner *via* the GnRHR to stimulate LRP production. Elevated LRP levels increase tumor cell interactions with laminin within the ECM, and this is an obligatory step in GnRH-II stimulation of MMP-2 production which plays a key role in ovarian cancer cell invasion (Fig. 4.13).
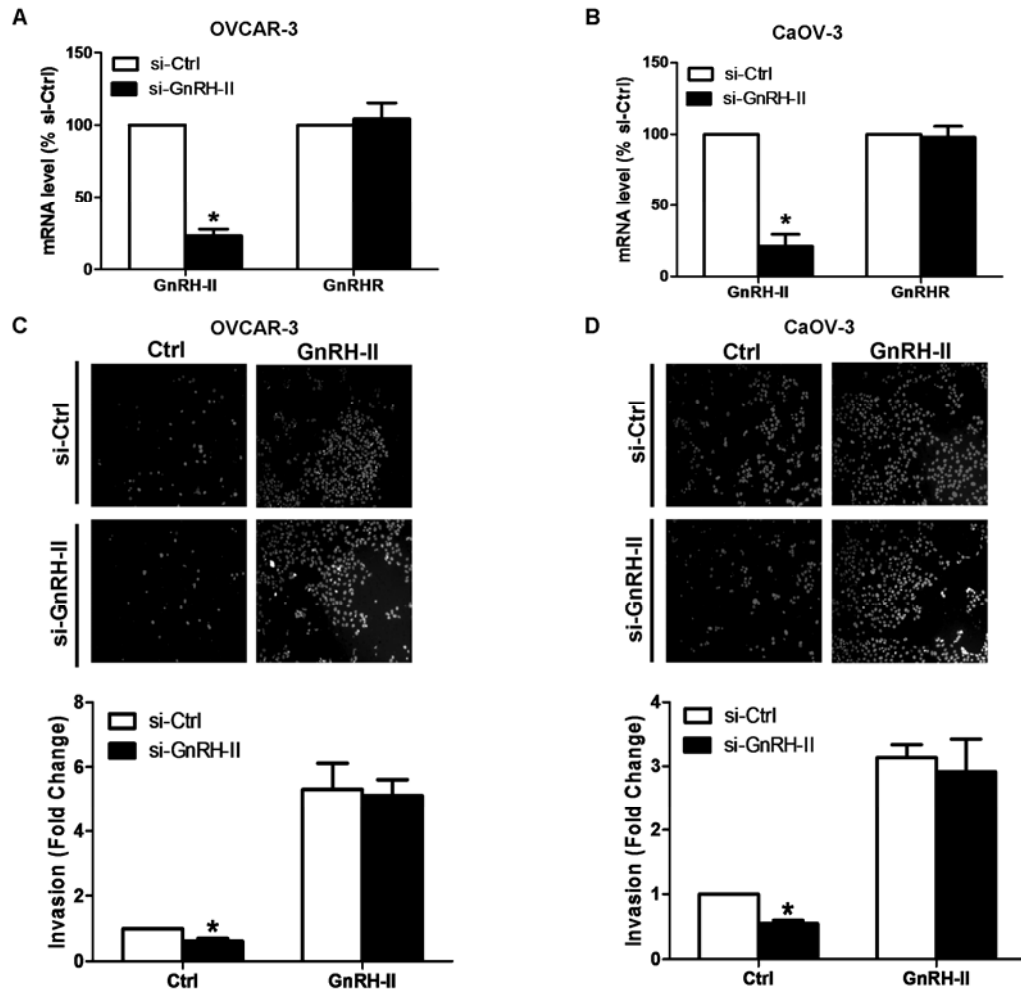
FIG. 4.1. **GnRH-II acts in an autocrine manner to enhance ovarian cancer cell invasion.** (A) OVCAR-3 and (B) CaOV-3 cells were treated with 100 nM GnRH-II siRNA (si-GnRH-II) or 100 nM control siRNA (si-Ctrl) for 24 h. In both cases, the mRNA levels of GnRH-II and GnRHR were measured by RT-qPCR and expressed as mean ± SEM of three independent experiments. *$p<0.05$ compared with control siRNA (si-Ctrl). (C, D) The cells were then seeded into Matrigel-coated transwells and cultured for 48 h in the presence of 10 nM GnRH-II. Non-invading cells were removed from the upper side of the filter and nuclei of invading cells were stained with Hoechst 33258. Upper panels show representative photomicrographs of cells attached to the lower membrane of the transwells in the invasion assays, while lower panels show the quantitative results of these assays, with results expressed as mean ± SEM of three independent experiments. *$p<0.05$ compared with control siRNA (si-Ctrl).
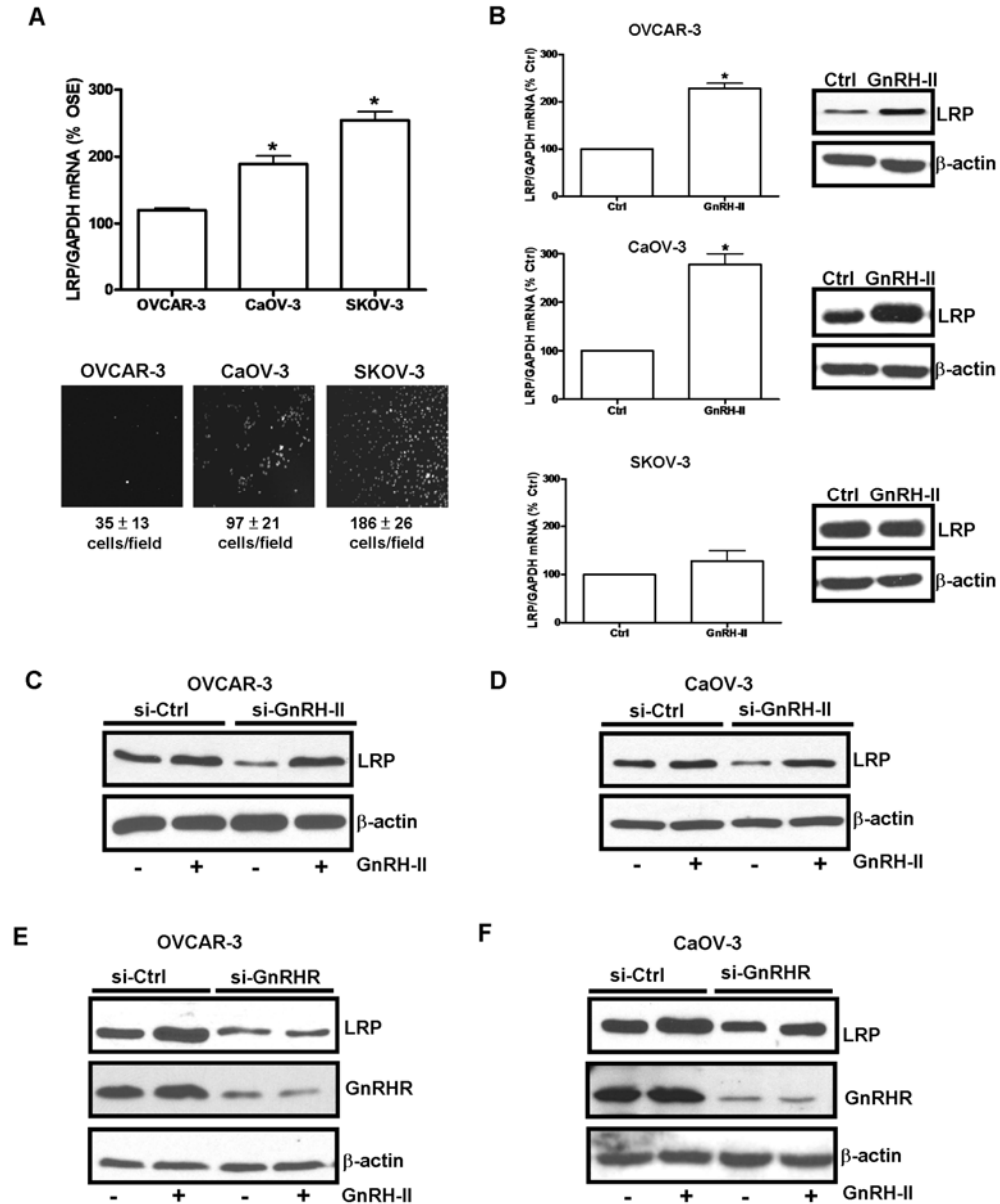
FIG. 4.2. GnRH-II induces the production of LRP in ovarian cancer cells. (A) LRP mRNA levels in OVCAR-3, CaOV-3 and SKOV-3 cells were measured by RT-qPCR and expressed relative to the levels in normal ovarian surface epithelial (OSE) cells. *$p<0.05$ compared with OSE. In parallel experiments, OVCAR-3, CaOV-3 and SKOV-3 cells were subjected to an invasion assay for 24 h. Lower panels show representative photomicrographs of cells attached to the lower membrane of transwells in the invasion assay. Below the photomicrographs, the numbers of invasive cells were quantified in 5 fields and expressed as means ± SEM of three independent experiments. (B) OVCAR-3, CaOV-3 and SKOV-3 cells were treated with 10 nM of GnRH-II and LRP mRNA (left panel) and LRP (right panel) levels were determined after 8h and 24 h, respectively. RT-qPCR results are expressed as mean ± SEM of three independent experiments. *$p<0.05$ compared with untreated control (Ctrl). (C, E) OVCAR-3 and (D, F) CaOV-3 cells were treated with 100 nM GnRH-II siRNA (si-GnRH-II), 100 nM GnRHR siRNA (si-GnRHR) or 100 nM control siRNA (si-Ctrl) for 24 h and then treated with 10 nM GnRH-II for a further 24 h. Cells were harvested and protein extracts were subjected to Western blotting and probed for LRP, GnRHR or β-actin as a normalization control.
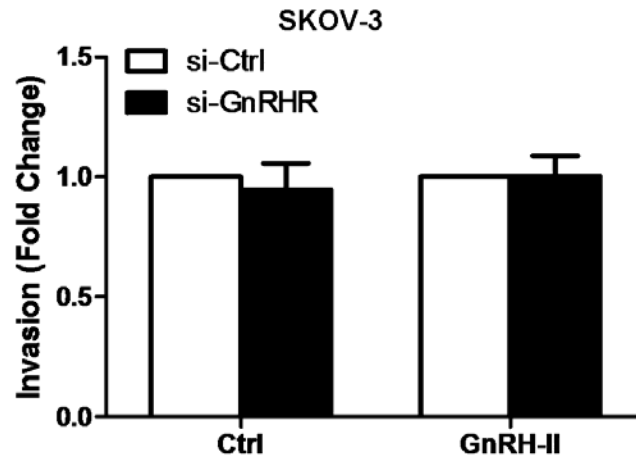
FIG. 4.3. GnRH-II treatment does not affect SKOV-3 cell invasiveness. SKOV-3 cells were treated with 100 nM GnRHR siRNA (si-GnRHR) or 100 nM control siRNA (si-Ctrl) for 24 h. The cells were then seeded into Matrigel-coated transwells and cultured for 24 h in the presence of 10 nM GnRH-II. Non-invading cells were removed from the upper side of the filter and nuclei of invading cells were stained with Hoechst 33258. Results are expressed as mean ± SEM of three independent experiments.
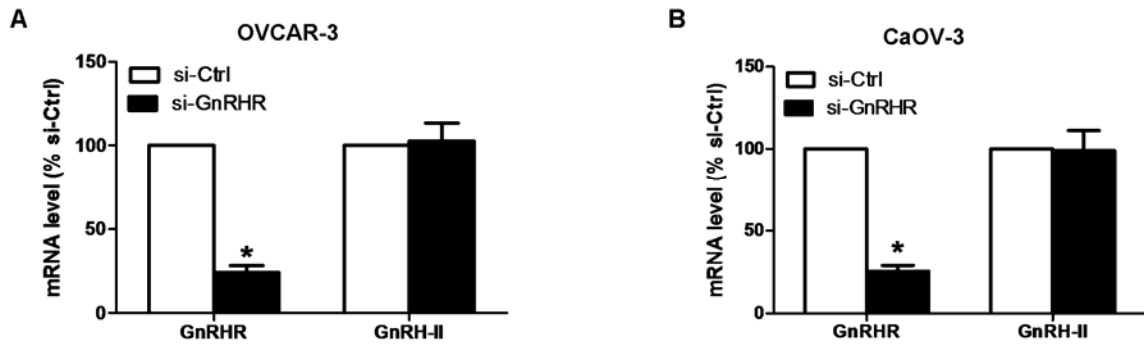
FIG. 4.4. Depletion of GnRHR with GnRHR siRNA does not influence GnRH-II mRNA levels in ovarian cancer cells. (A) OVCAR-3 and (B) CaOV-3 cells were treated with 100 nM GnRHR siRNA (si-GnRHR) or 100 nM control siRNA (si-Ctrl) for 24 h. Total RNA from treated cells was used to prepare cDNA for RT-qPCR analysis to evaluate the efficiency of GnRHR siRNA and the effects of GnRHR on GnRH-II mRNA levels. RT-qPCR results are expressed as mean ± SEM of three independent experiments. *$p<0.05$ compared with control siRNA (si-Ctrl).
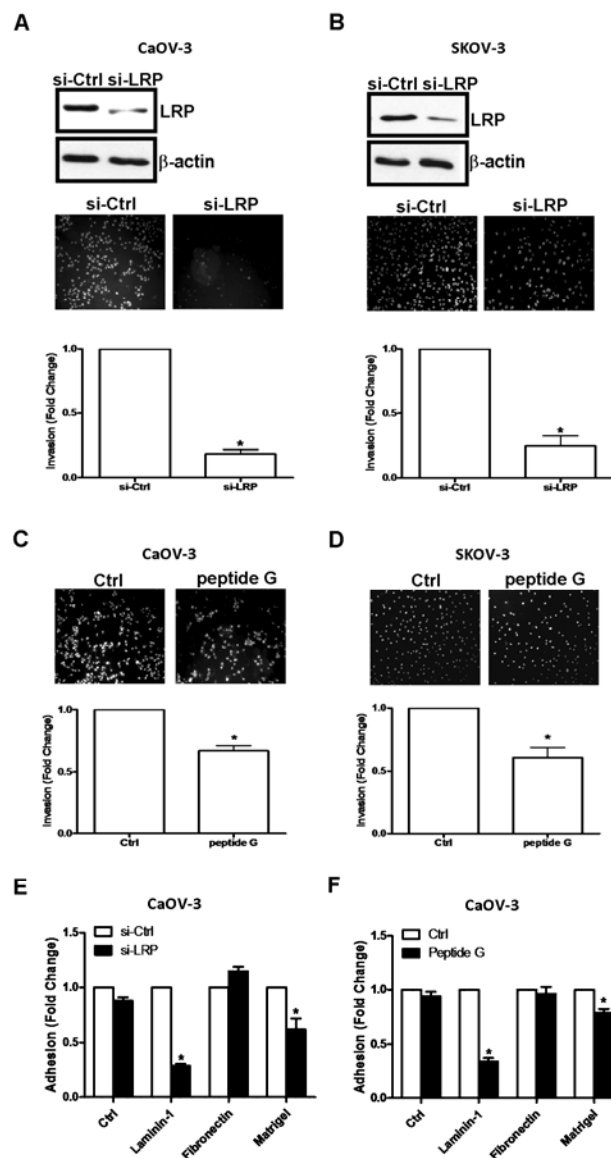
FIG. 4.5. LRP mediates the invasive potential of ovarian cancer cells. (A) CaOV-3 and (B) SKOV-3 cells were treated with 75 nM LRP siRNA (si-LRP) or 75 nM control siRNA (si-Ctrl) prior to invasion assays for 48 h (CaOV-3 cells) or 24 h (SKOV-3 cells). In both cases, the efficiency of LRP siRNA was verified by Western blotting (upper panels). Synthetic peptide G (1 μg) was pre-incubated with the Matrigel-coated transwells for 30 min before (C) CaOV-3 and (D) SKOV-3 cells were subjected to invasion assays. In A,B,C and D, representative photomicrographs of cells attached to the lower membrane of the transwells in the invasion assays are shown, and the lower panels show the quantitative results of these assays, with results expressed as mean ± SEM of three independent experiments. *$p<0.05$ compared with control siRNA (si-Ctrl) in A and B or untreated control (Ctrl) in C and D. (E) CaOV-3 cells were treated with si-LRP or si-Ctrl prior to adhesion assays for 8 h on uncoated (Ctrl) tissue culture plates, or plates coated with laminin-I, fibronectin or Martigel. Results are expressed as mean ± SEM of three independent experiments. *$p<0.05$ compared with control siRNA (si-Ctrl). (F) Peptide G (1 μg) was pre-incubated with the uncoated (Ctrl) or coated tissue culture plates (i.e., with fibronectin, laminin-1, or Matrigel) for 30 min before CaOV-3 cells were tested in an adhesion assay for 8 h. Results are expressed as mean ± SEM of three independent experiments. *$p<0.05$ compared with untreated control (Ctrl).
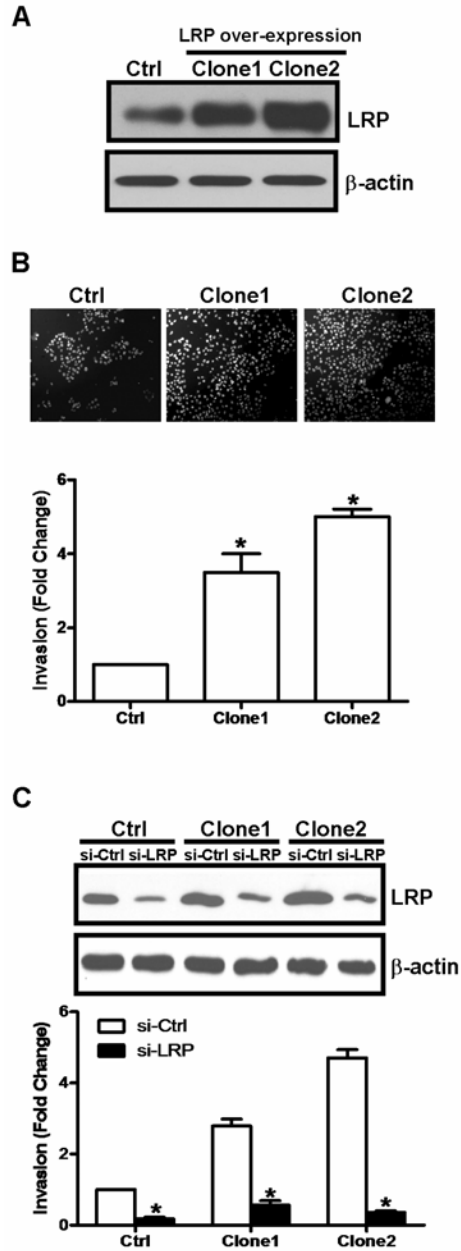
FIG. 4.6. Over-expressing LRP up-regulates ovarian cancer cell invasiveness. Over-expressing LRP up-regulates ovarian cancer cell invasiveness. CaOV-3 cells were stably transfected with expression vectors for wild-type LRP or eGFP as a control (Ctrl). (A) Over-expression of LRP in two independent clones (i.e. Clones 1 and 2) was verified by Western blotting. (B) In parallel experiments, these stably transfected CaOV-3 cells were subjected to invasion assays for 48 h. The upper panel shows representative photomicrographs of cells attached to the lower membrane of the transwells in the invasion assays, and the lower panels show the quantitative results of these assays, with results expressed as mean ± SEM of three independent experiments. *$p<0.05$ compared with control (Ctrl). (C) LRP over-expressing cells (Clones 1 and 2) and control (Ctrl) cells were treated with 75 nM LRP siRNA (si-LRP) or 75 nM control siRNA (si-Ctrl) prior to invasion assays for 48 h. The efficiency of LRP siRNA in these LRP over-expressing clones were verified by Western blotting of LRP (upper panel). Results of invasion assays are expressed as mean ± SEM of three independent experiments. *$p<0.05$ compared with the control siRNA (si-Ctrl).
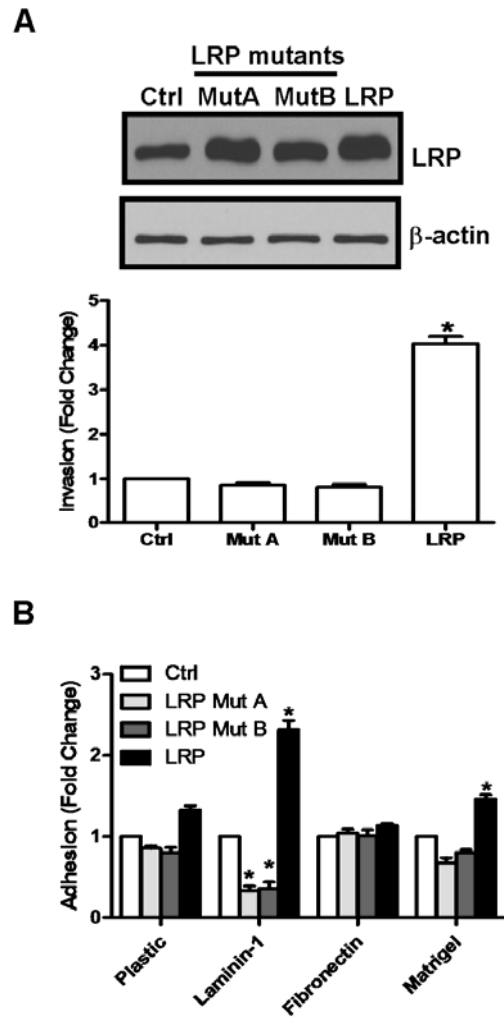
FIG. 4.7. The laminin binding domain of LRP plays an important role in ovarian cancer cell invasion. (A) CaOV-3 cells were stably transfected with wild-type LRP (LRP) or two different mutated LRP expression vectors (i.e. Mut A, Mut B). The relative LRP levels in these cells, and cells expressing eGFP as a control, (Ctrl) were compared by Western blotting (upper panel), and invasion assays using these cells were performed for 48 h (lower panel). (B) In parallel experiments, these stably transfected cells were also subjected to adhesion assays for 8 h. In both A and B, results are expressed as mean ± SEM of three independent experiments. *$p<0.05$ compared with the control cells (Ctrl).
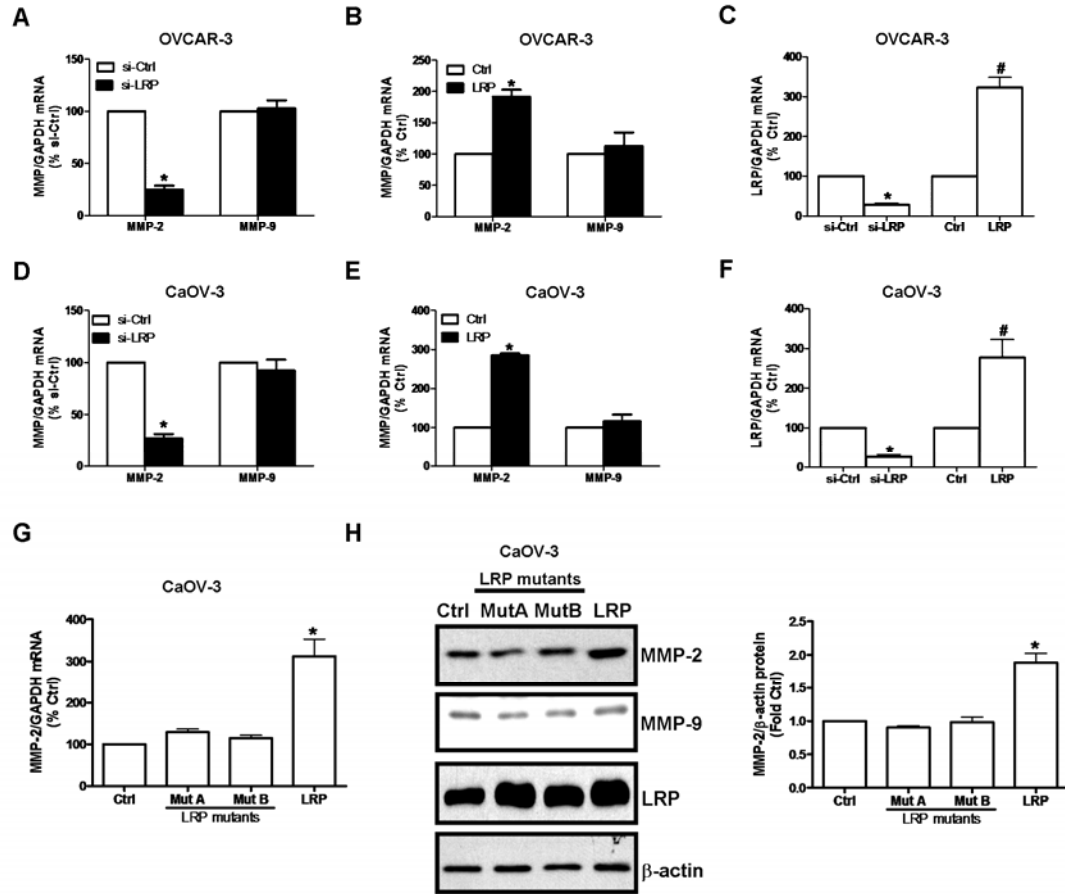
FIG. 4.8. LRP regulates MMP-2 production in ovarian cancer cells. OVCAR-3 and CaOV-3 cells were treated with (A, D) 75 nM LRP siRNA (si-LRP) or 75 nM control siRNA (si-Ctrl), or (B, E) transiently transfected with 1 μg wild-type LRP expression vector or 1 μg eGFP expression vector as a control. (C, F) In both cases, the efficiency of LRP siRNA and over-expression of LRP in these cells were verified by RT-qPCR measurements of LRP mRNA levels. Total RNA from transfected cells was used to prepare cDNA for RT-qPCR analysis to evaluate the effects of LRP on MMP-2 and MMP-9 mRNA levels, which are expressed in A and D as a percentage over control siRNA (si-Ctrl) values, or in B and E as a percentage of corresponding values in the control (Ctrl) cells. (G) Total RNA of CaOV-3 cells stably over-expressing mutated LRP (i.e. Mut A and Mut B), eGFP control vector (Ctrl) and wild-type LRP (LRP) was isolated and subjected to RT-qPCR to measure the MMP-2 and MMP-9 mRNA levels expressed as percentage over cells expressing the control vector (Ctrl). Results are expressed as the mean ± SEM of three independent experiments. *$p<0.05$ compared with respective controls. (H) In parallel experiments, protein lysates from these stably over-expressing cells were harvested and subjected to Western blotting for their relative MMP-2, MMP-9 and LRP protein levels or β-actin as a normalization control. The Western blotting data are expressed as mean ± SEM of three independent experiments (right panel). *$p<0.05$ compared with the control cells (Ctrl).
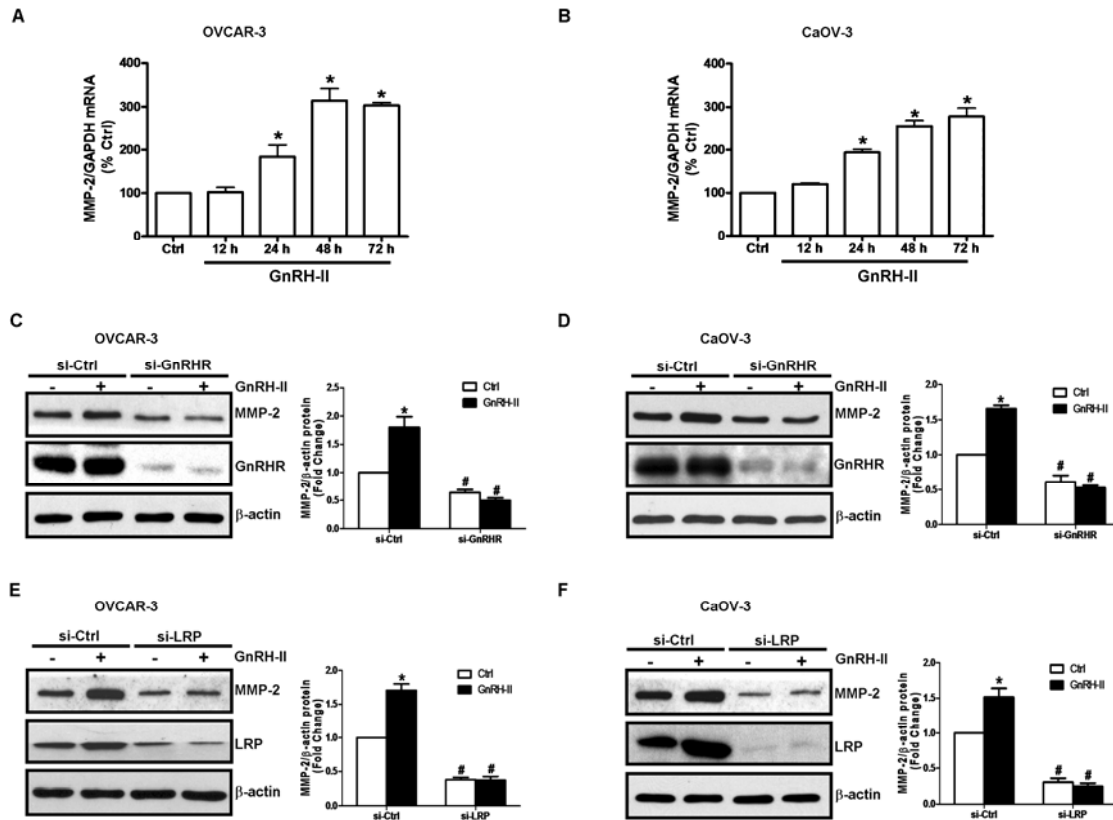
FIG. 4.9. LRP mediates the GnRH-II-induced production of MMP-2 by ovarian cancer cells. (A) OVCAR-3 and (B) CaOV-3 cells were treated with 10 nM GnRH-II for up to 72 h. Total RNA from treated cells was used to prepare cDNA for RT-qPCR analysis to evaluate the effect of GnRH-II on MMP-2 mRNA levels. RT-qPCR results are expressed as mean ± SEM of three independent experiments. *p<0.05 compared with untreated control (Ctrl). (C and D) OVCAR-3 and CaOV-3 cells were treated with 100 nM GnRHR siRNA (si-GnRHR) or 100 nM control siRNA (si-Ctrl) for 24 h and then treated with 10 nM GnRH-II for 48 h. (E and F) OVCAR-3 and CaOV-3 cells were treated with 75 nM LRP siRNA (si-LRP) or 75 nM control siRNA (si-Ctrl) for 24 h, and then treated with 10 nM GnRH-II treatment for 48 h. In C-F, cells were harvested and protein extracts were subjected to Western blotting with antibodies against MMP-2, GnRHR, LRP or β-actin as a normalization control. Left panel showed representative Western blots and right panel showed the quantitative results of three independent experiments.
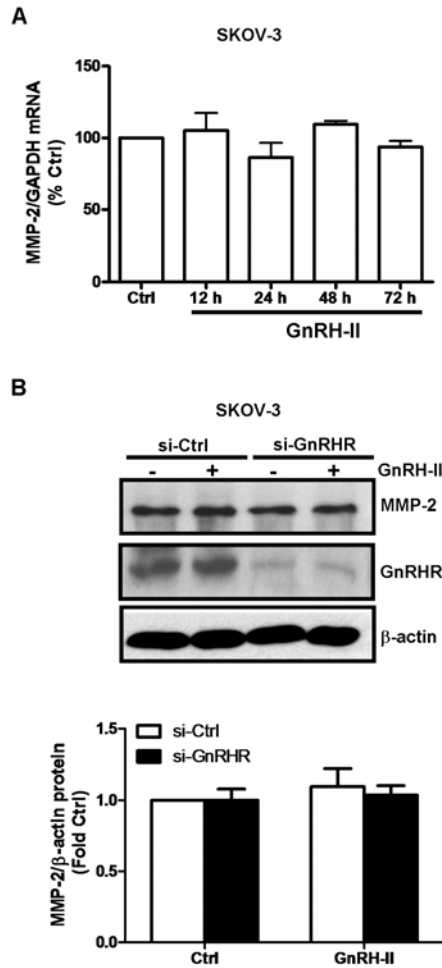
FIG.4.10. GnRH-II treatment does not affect SKOV-3 cell MMP-2 levels. (A) SKOV-3 cells were treated with 10 nM GnRH-II for up to 72 h. Total RNA from treated cells was used to prepare cDNA for RT-qPCR analysis to evaluate the effect of GnRH-II on MMP-2 mRNA levels. RT-qPCR results are expressed as mean ± SEM of three independent experiments. (B) SKOV-3 cells were treated with 100 nM GnRHR siRNA (si-GnRHR) or 100 nM control siRNA (si-Ctrl) for 24 h and then treated with 10 nM GnRH-II for 24 h. Treated cells were harvested and protein extracts were subjected to Western blotting with antibodies against MMP-2, GnRHR or β-actin as a normalization control.
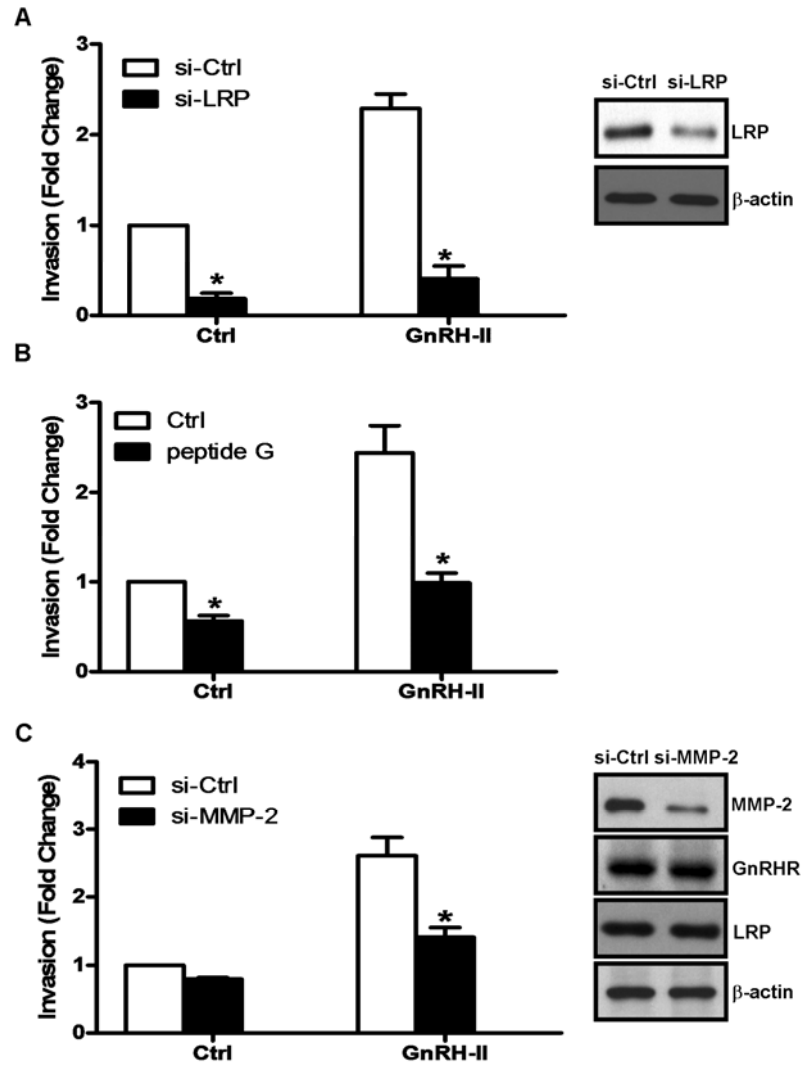
FIG. 4.11. LRP and MMP-2 are key mediators of GnRH-II enhanced invasion in ovarian cancer cells. (A) CaOV-3 cells were treated with 75 nM LRP siRNA (si-LRP) or 75 nM control siRNA (si-Ctrl) for 24 h, and with 10 nM GnRH-II for a further 48 h during an invasion assay. (B) In parallel experiments, 1 μg of peptide G was pre-incubated with Matrigel-coated transwells for 30 min prior invasion assay in the presence of 10 nM GnRH-II for 48 h. (C) CaOV-3 cells were treated with 75 nM MMP-2 siRNA (si-MMP-2) or 75 nM control siRNA (si-Ctrl) for 24 h, prior an invasion assay in the presence of 10 nM GnRH-II for 48 h. Results are expressed as mean ± SEM of three independent experiments. *$p<0.05$ compared with control siRNA (si-Ctrl) in A and C or untreated control (Ctrl) in B. The efficiency of LRP siRNA and MMP-2 siRNA were verified by Western blot analysis of LRP (right panels in A and C). In parallel experiments, si-MMP-2 treated cells were harvested and protein extracts were subjected to Western blotting with antibodies against LRP, GnRHR or β-actin as a normalization control.
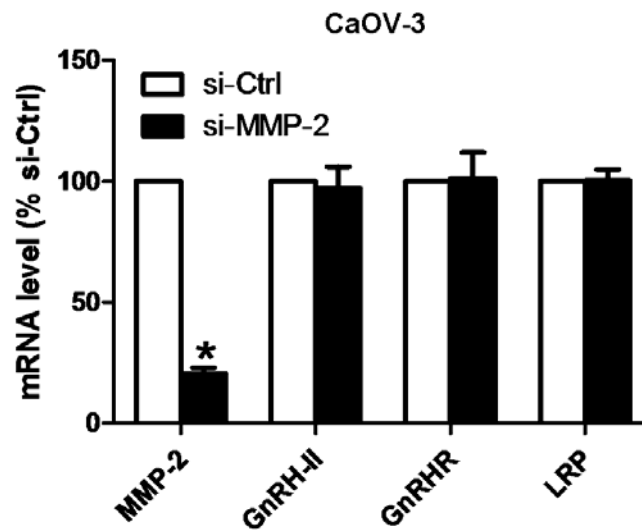
FIG. 4.12. Depletion of MMP-2 levels with siRNA does not affect GnRH-II, GnRHR or LRP mRNA levels in SKOV-3 cells. SKOV-3 cells were treated with 75 nM MMP-2 siRNA (si-MMP-2) or 75 nM control siRNA (si-Ctrl) for 24 h. Total RNA from treated cells was used to prepare cDNA for RT-qPCR analysis to evaluate the efficiency of MMP-2 siRNA and the effects of MMP-2 on GnRH-II, GnRHR and LRP mRNA levels. RT-qPCR results are expressed as mean ± SEM of three independent experiments. *$p < 0.05$ compared with control siRNA (si-Ctrl).
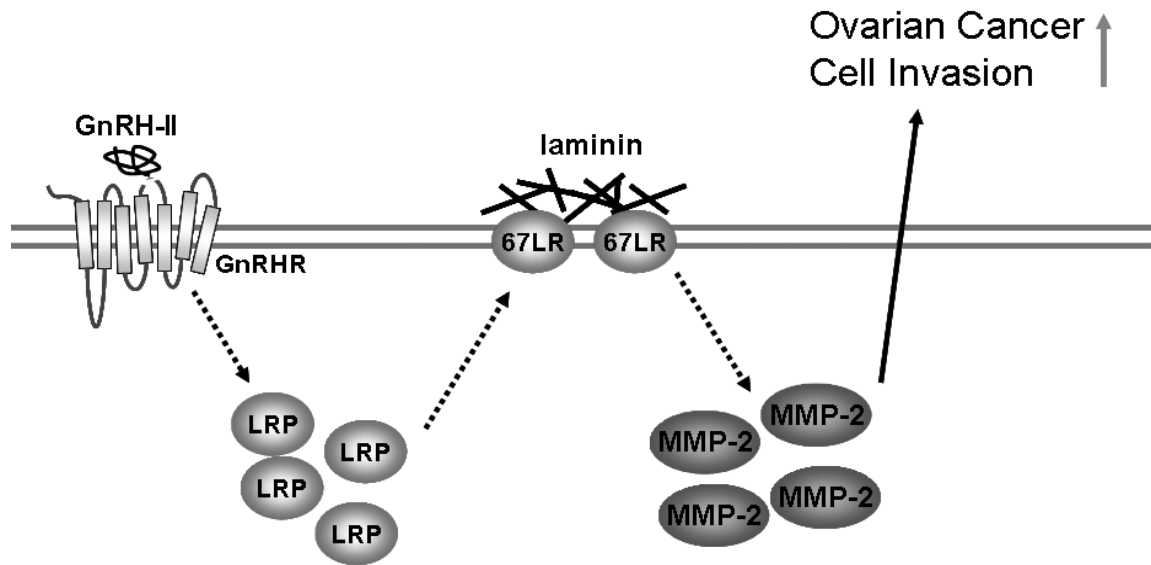
FIG. 4.13. Proposed model for 67LR as a key mediator in GnRH-II-enhanced ovarian cancer cell invasion. GnRH-II/GnRHR acts in an autocrine manner to increase the amount of 67LR in ovarian cancer. This leads to an increased between the interaction of laminin with cancer cells, thereby up-regulates the production of MMP-2. Increased production of MMP-2 contributes to the GnRH-II-induced ovarian cancer cell invasive potential. 67LR, 67 kDa non-integrin laminin receptor; LRP, 37 kDa non-integrin laminin receptor precursor; GnRHR, GnRH receptor; MMP-2, matrix metalloproteinase-2.

CHAPTER 5. Gonadotropin-releasing hormone-II increases membrane type I metalloproteinase production *via* β-catenin signaling in ovarian cancer cells

5.1 Introduction

Gonadotropin-releasing hormone-II (GnRH-II) acts on a variety of extrapituitary tissues and tumor cells (67, 292), including ovarian cancers. The widespread presence (>80%) of the GnRH receptor (GnRHR) in ovarian carcinomas (280) and higher levels of GnRH-II mRNA in malignant ovarian tumors, as compared with benign ovarian tumors (139), underlies the importance of understanding the function of GnRH-II in ovarian cancer. Previously, we have shown that GnRH-II acts in an autocrine manner to enhance ovarian cancer cell invasion (53), but the molecular mechanisms that mediate this action of GnRH-II are poorly understood.

The GnRHR is a G protein coupled receptor that binds GnRH-I and GnRH-II (53). Binding of these ligands to the GnRHR results in the activation of several downstream signaling molecules, such as mitogen activated protein kinase (MAPK), phosphatidylinositol-3-kinase (PI3K), and nuclear factor kappa B (52, 53). Recently, Wnt/β-catenin signaling has been shown to play an essential role in transducing the GnRHR signal in gonadotropes, and that this involves an accumulation of β-catenin in the nucleus (92, 93). This is important because β-catenin acts as a signaling molecule in the Wnt/β-catenin pathway (85-87), and many Wnt/β-catenin-target genes contribute to the progression and metastasis of a variety of tumor types (88, 293, 294). In the absence of Wnt ligand, the cellular levels of β-catenin are kept low by a destruction complex that includes glycogen synthase kinase 3β (GSK3β), which phosphorylates β-catenin and targets it for degradation (295). Stimulation of the Wnt pathway leads to the phosphorylation of GSK-3β, which prevents it from phosphorylating β-catenin, and this thereby stabilizes and releases β-catenin for translocation to the nucleus (296).

Metastasis is a complex process that starts with the dissemination of cancer cells from a primary tumor to distant tissues. Along the metastatic cascade, tumor cells interact with and remodel the extracellular matrix (ECM), and a variety of matrix metalloproteinases (MMPs) are key players in the process of tumor cell invasion (143). Membrane type I matrix metalloproteinase (MT1-MMP) has been directly correlated with enhanced cell migration and is generally considered pro-invasive and pro-tumorigenic (297). Ovarian cancer tissue microarray (298) studies have shown that MT1-MMP is expressed in all four subtypes of epithelial ovarian carcinoma (i.e., serous, mucinous, endometroid, clear cell), and high MT1-MMP levels in epithelial ovarian carcinoma have been reported to be indicative of poor prognosis (299, 300). In addition, MT1-MMP synthesis is known to be under the regulation of Wnt/β-catenin signaling in mesenchymal stem cells and gastric cancer cells (301, 302), and *in vitro* studies of a variety of ovarian cancer cell lines, including OVCAR-3 cells, have indicated that MT1-MMP levels play an important role in ovarian cancer cell invasiveness (303, 304).

We now demonstrate that GnRH-II induces the nuclear accumulation of β-catenin *via* PI3K/Akt signaling in ovarian cancer cell lines that express the GnRHR, and that β-catenin up-regulates MT1-MMP production and contributes to GnRH-II-enhanced ovarian cancer cell invasion.

5.2 Materials and methods

Cells and cell culture

The human ovarian adenocarcinoma cell lines, OVCAR-3 and CaOV-3, were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were maintained in M199/MCDB105 (Invitrogen Inc., Burlington, ON, Canada) supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories Inc., Logan, UT, USA). Cultures were maintained at

37 C in a humidified atmosphere of 5% CO_2. The cells were sub-cultured when they reached about 90% confluence using a trypsin/EDTA solution (0.05% trypsin, 0.5 mM EDTA). Both ovarian cancer cell lines express GnRHR (52) and have been shown to response to GnRH-II treatment (53).

Antibodies and reagents

GnRH-II analog (DArg6-Azagly10-GnRH-II) was purchased from Bachem (Belmont, CA). The polyclonal MT1-MMP antibody was obtained from Abcam (Cambridge, MA; cat# ab3644). The monoclonal GnRHR antibody was obtained from Neomarkers (Fremont, CA; cat# MS-1139-P). The polyclonal antibodies for phosphorylated Akt (cat# 9271s), total Akt (cat# 9272) and phosphorylated GSK3β (cat# 9336s) were obtained from Cell Signaling Technology (Danvers, MA). The monoclonal β-catenin antibody was obtained from BD Bioscience (San Jose, CA; cat# 610154). The polyclonal β-actin antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA; cat# C-11), as was the horseradish peroxidase-conjugated donkey anti-goat IgG. Horseradish peroxidase-conjugated goat anti-rabbit IgG and goat anti-mouse IgG were obtained from Bio-Rad Laboratories (Hercules, CA).

Nuclear extraction and Western blotting

Briefly, cells were washed with ice-cold PBS and harvested with 1 ml solution A (10 mM Hepes, pH 7.9, 10 mM KCl, 10 mM EDTA, 0.5 mM dithiothreitol, 1 μg/ml aprotinin, and 1 μg/ml proteinase inhibitor cocktail). Cell lysates were transferred to 1.5 ml centrifuge tubes and placed in an orbital rocker for 10 min at 4 C. Nuclear pellets were obtained by centrifugation at 14,000 g at 4 C for 10 min, re-suspended in solution B (100 mM HEPES, pH 7.9, 2 M NaCl, 5 mM EDTA, 50% Glycerol) and placed in an orbital rocker for 2 h at 4 C. After centrifugation at

14,000 g at 4 C for 5 min, supernatants containing the nuclear protein extracts were removed. The nuclear extracts were then subjected to electrophoresis on an 8% SDS-PAGE gel and Western blotted for detection with appropriate antibodies. Total cell lysates were also prepared using Cell Lysis Buffer (Cell Signaling) and subjected to Western blotting.

Luciferase assay and siRNA transfection

To monitor the transcriptional activity of β-catenin, cells were grown in standard M199/MCDB105 culture medium containing FBS and co-transfected with 0.5 μg of the TOPFLASH luciferase reporter plasmid (Millipore, Temecula, CA) and 0.25 μg of a RSV-*lacZ* plasmid (53). After 6 h, 2 ml culture medium with 0.5% FBS was added and the cells were further incubated overnight (18 h). The culture medium was then removed and the cells were pre-treated with 10 μM LY294002 and then with 10 nM GnRH-II treatment in culture medium containing 0.5% FBS for 24 h. Cellular lysates were collected with 150 μl reporter lysis buffer (Promega, Madison, WI) and assayed for luciferase activity. The β-Galactosidase Enzyme Assay System (Promega) was used to measure β-galactosidase expression from the (RSV)-*lacZ* plasmid, and promoter activities were expressed as luciferase activity/β-galactosidase activity.

siRNA transfections were carried out using Lipofectamine RNAiMAX Reagent (Invitrogen) following the manufacturer's protocol. Briefly, 5×10^5 cells were seeded into six-well tissue culture plates one day prior to transfection with 100 nM GnRHR siRNA (si-GnRHR), 50 nM β-catenin siRNA (si-β-catenin), 50 nM MT1-MMP siRNA (si-MT1-MMP) or a non-targeting control siRNA (si-Ctrl) (Dharmacon, Inc., Lafayette CO). After 6 h, the culture medium in each well was replaced with 2 ml culture medium containing 0.5% FBS, and the cells were further incubated overnight (18 h). The culture medium was then removed and the

cells were treated with 10 nM GnRH-II in culture medium containing 0.5% serum for the times indicated.

Invasion assay

Transwell cell culture inserts (8 μm pore size, 24-wells, BD Biosciences) were coated with 40 μl of 1 mg/ml growth-factor reduced Matrigel (BD Biosciences). Cells in M199/MCDB105 medium supplemented with 0.5% FBS were incubated for 48 h against a gradient of 10% FBS for OVCAR-3 cells and CaOV-3 cells. Cells on the lower side of the membrane were fixed with ice-cold methanol, stained with Hoechst 33258 (Sigma), and the number of nuclei was counted using a Zeiss Axiophot epifluorescent microscope equipped with a digital camera and Northern Eclipse 6.0 software from Empix Imaging (Empix Imaging, Mississauga, ON). Individual experiments were run with duplicate inserts and five microscopic fields were counted per insert.

Data analysis

Results are shown as the mean ± SEM of three independent experiments. Data were analyzed by one-way ANOVA followed by Tukey test using PRISM software (GraphPad Software Inc., San Diego, CA, USA). Values were considered significantly different from each other at $p < 0.05$.

5.3 Results

GnRH-II activates PI3K/Akt and β-catenin signaling pathways in ovarian cancer cells

We first set out to identify downstream signaling events in ovarian cancer cells after treatment with GnRH-II. As shown in Figure 5.1A and B, when OVACAR-3 and CaOV-3

ovarian cancer cells were treated with 10 nM GnRH-II for different times (5, 15, 30, 60 min), there was a transient increase in the phosphorylation of both Akt and GSK3β. Although the phosphorylation of Akt decreases in GnRH-II-treated cells after 30 min, we observed a progressive increase in GSK3β phosphorylation up to 60 min after GnRH-II treatment (Fig. 5.1A and B). This is important because the phosphorylation status of GSK3β mediates the stabilization and accumulation of nuclear β-catenin (296). In this regard, when nuclear lysates of the GnRH-II-treated OVCAR-3 and CaOV-3 cells were subjected to Western blotting, it was also apparent that GnRH-II promotes the accumulation of β-catenin in the nucleus for at least 60 min (Fig. 5.1C and D).

To examine whether the GnRH-II-induced β-catenin nuclear translocation is a GnRHR-dependent mechanism, OVCAR-3 cells and CaOV-3 cells were treated overnight with GnRHR siRNA prior treatment with GnRH-II for 30 min. Nuclear lysates of these treated cells were then prepared to monitor the accumulation of β-catenin in the nucleus by Western blotting. This revealed that depletion of GnRHR in these cell lines attenuated the GnRH-II-induced β-catenin accumulation in the nucleus (Fig. 5.1E and F).

To determine whether GnRH-II treatment of OVCAR-3 or CaOV-3 cells induced the activity of β-catenin as a transcription factor, we transfected these cancer cells with the TOPFLASH luciferase reporter gene and then treated them with GnRH-II for 24 h before measurement of luciferase activity. As shown in Figure 5.2A and B, GnRH-II induces 2 - 3 fold increases in luciferase activity in OVCAR-3 cells and CaOV-3 cells. In addition, this induction was completely inhibited by pre-treatment with a PI3K/Akt signaling inhibitor, LY294002 (Fig. 5.2A and B), suggesting that GnRH-II activates the PI3K/Akt pathway and that this induces β-catenin signaling in these ovarian cancer cells. Under similar conditions, cells pre-treated with

LY294002 attenuated GnRH-II-induced invasion in OVCAR-3 and CaOV-3 cells (Fig. 5.2C and D).

To verify whether activation of PI3K/Akt signaling results in the phosphorylation of GSK3β, and thereby contributes to GnRH-II-induced β-catenin nuclear translocation, OVCAR-3 (Fig. 5.3A) and CaOV-3 cells (Fig. 5.3B) were pre-treated with LY294200 for 30 min prior 10 nM GnRH-II treatment. Treated cells were harvested and subjected to Western blotting to determine the phosphorylation status of GSK3β. The results suggest that GnRH-II-induced PI3K/Akt signaling involves an increase in GnRH-II mediated GSK3β phosphorylation.

GnRH-II up-regulates MT1-MMP production in a GnRHR-dependent manner

The MT1-MMP has been shown to play a role in ovarian cancer cells invasion (304). When OVCAR-3 cells and CaOV-3 cells were treated with GnRH-II for 24 h and 48 h, we observed significant increases in their MT1-MMP levels (Fig. 5.4A and B). To examine whether GnRH-II acts through the GnRHR to exert this effect in OVCAR-3 and CaOV-3 cells, the cells were pre-treated with GnRHR siRNA to knock down the endogenous levels of GnRHR. In cells depleted of GnRHR, GnRH-II treatment failed to increase MT1-MMP levels (Fig. 5.4C and D).

The β-catenin signaling pathway increases MT1-MMP production in ovarian cancer cells

It is known that β-catenin signaling directly influences human *MT1-MMP* gene transcription (305). To examine whether *MT1-MMP* is a downstream target of β-catenin signaling in ovarian cancer cells, OVCAR-3 cells (Fig. 5.5A) and CaOV-3 cells (Fig 5.5B) were treated with lithium chloride (LiCl) for 24 h. We used LiCl for this purpose because it is known to increase the phosphorylation of GSK3β and to thereby facilitate the nuclear translocation of β-catenin (306) in numerous cell types (307, 308). When cell lysates of LiCl-treated cells were

prepared and subjected to Western blotting, we observed an increase in MT1-MMP levels (Fig. 5.5A and B), which would be consistent with an activation of β-catenin

To further assess the role of β-catenin in terms of GnRH-II-induced MT1-MMP production in ovarian cancer cells, a specific siRNA was used to knock down β-catenin levels in OVCAR-3 and CaOV-3 cells (Fig. 5.5C and D). In CaOV-3 cells, we consistently observed an immunoreactive band that migrates with a higher molecular size than β-catenin. Since the intensity of this immunoreactive band was not influenced by treatment of the cells with a β-catenin siRNA (Fig. 5.5D), we conclude that it represents a non-specific interaction between the β-catenin antibody and another protein. As shown in Figure 5.5C and D, depletion of β-catenin abolished the GnRH-II-induced MT1-MMP levels, which indicates that GnRH-II-activated β-catenin signaling up-regulates MT1-MMP production in these ovarian cancer cells. In addition, this increase in MT1-MMP synthesis was attenuated when we blocked PI3K/Akt signaling by pre-treatment of OVCAR-3 (Fig. 5.5E) and CaOV-3 cells (Fig. 5.5F) with LY294200 for 30 min prior GnRH-II treatment, suggesting that GnRH-II activates PI3K/Akt signaling and increases β-catenin accumulation in the nucleus, and that these events participate in up-regulating MT1-MMP production in ovarian cancer cells.

β-Catenin enhances MT1-MMP production and contributes to GnRH-II induced ovarian cancer cell invasion

Our finding that β-catenin signaling stimulates MT1-MMP production after GnRH-II treatment suggests that it plays a role in GnRH-II-induced ovarian cancer cell invasion. Thus, we employed a siRNA strategy to explore the role of β-catenin and MT1-MMP in GnRH-II induced invasion in OVCAR-3 cells and CaOV-3 cells. Both cell lines were treated with β-catenin siRNA (Fig. 5.6A and B) or MT1-MMP siRNA (Fig. 5.6C and D), prior to an invasion

assay. As compared with OVCAR-3 cells and CaOV-3 cells treated with control siRNA, the corresponding cells depleted of β-catenin or MT1-MMP were resistant to a GnRH-II induced increase in their invasive potential.

5.4 Discussion

Recent studies have demonstrated that activation of GnRHR initiates Wnt/β-catenin signaling and indirectly promotes GnRH-induced LHβ-subunit gene expression in gonadotropes (92). Interestingly, the initiation of Wnt signaling by GnRH-I and GnRH-II in the human embryonic kidney 293 cell line can be manipulated by over-expressing GnRHR (93), suggesting that the involvement of GnRH/GnRHR signaling and the β-catenin pathway extends to extra-pituitary cell types. In this regard, we now provide evidence that GnRH-II activates β-catenin signaling and promotes the invasive properties of GnRHR positive ovarian cancer cells.

It is well known that GSK3β is a negative regulator of Wnt signaling (94), and that activation of the PI3K/Akt pathway regulates the phosphorylation of GSK3β, which acts to enhance β-catenin nuclear translocation (309). Likewise, we have found that GnRH-II induced PI3K/Akt signaling stimulates the phosphorylation of GSK3β and that GnRH-II treatment fails to enhance the transcriptional activity of β-catenin, as measured in a TOPflash luciferase reporter gene assay (293), when GSK3β phosphorylation is blocked. These data imply that PI3K/Akt signaling plays a key role in the β-catenin mediated effects of GnRH-II in ovarian cancer cells.

The Wnt/β-catenin signaling pathway has been well defined in terms of the physiologic regulation of many cells (310-312), and the progression of different cancer cell types (313). There are two ways that Wnt/β-catenin signalling can contribute to the oncogenic phenotype, both of which enhance β-catenin translocation and accumulation in the nucleus, where it

associates with T-cell factor/lymphoid enhancer factor (TCF/LEF) and thereby initiates transcription of pro-invasive target genes (88). In the first instance, mutation of a tumor suppressor gene, adenomatous polyposis coli (APC), disrupts the normal formation of the APC/GSK3β/β-catenin complex in the cytoplasm, and leads to aberrant translocation of β-catenin to the nucleus (314). In the second scenario, mutations in β-catenin gene result in the constitutive accumulation of β-catenin in the cell nucleus (315). While there is no evidence for an etiologic role for APC in ovarian cancer (316), there is compelling evidence for altered Wnt signaling and increased nuclear β-catenin levels in different ovarian carcinoma subtypes (317, 318), and that this contributes to ovarian cancer progression (96). In particular, a significant correlation has been reported between nuclear β-catenin accumulation and the grade of serous carcinomas, which shows a trend toward decreased survival (104). In line with these reports, our findings suggest that β-catenin signaling plays an important role in GnRH-II mediated increases in ovarian cancer cell invasion.

We therefore considered it important to examine potential targets of β-catenin signaling that might contribute to the increased invasive properties of ovarian cancer cells after treatment with GnRH-II, and we focussed our attention on MT1-MMP because it is frequently expressed in a variety of human tumors (297), including ovarian cancer (303), and because there is a well defined TCF/LEF binding site for β-catenin in the *MT1-MMP* promoter (319). Our experiments indicate that GnRH-II stimulates MT1-MMP synthesis in ovarian cancer cells in a GnRHR-dependent manner, and that this involves the activation of PI3K/Akt signaling, which leads to an increase in the transcriptional activity of β-catenin. In line with observations that altered levels of MT1-MMP are associated with cancer cell invasion (297), our data also show that siRNA-mediated depletion of MT1-MMP attenuates GnRH-II enhanced ovarian cancer cell invasion by ~ 50%. Although MT1-MMP can act as a processing enzyme for CD44 and collagen I (156),

one of its primary functions is to cleave the proMMP-2 zymogen into its enzymatically active form (158, 320) which functions to promote cancer cell motility (172, 321, 322). We consider this to be particularly important because we have also recently found that GnRH-II indirectly increases *MMP-2* expression (Poon *et al.*, unpublished) in ovarian cancer cells. Moreover, since β-catenin activation does not play a role in GnRH-II mediated increases in *MMP-2* expression (data not shown), we conclude that the autocrine actions GnRH-II/GnRHR activate multiple independent pathways which converge and additively contribute to the over-production of MMP-2.

The ECM is generally characterized as either a basement membrane or stromal/interstitial type. Matrigel is a synthetic ECM comprising an Engelbreth-Holm-Swarm mouse sarcoma extract, which includes laminin, collagen IV and entactin, and is thought to resemble the basement membrane (323). By contrast, collagen I is a major component of the stromal/interstitial type of ECM, and collagen I coated transwells have recently been suggested be a more appropriate model for studies of cancer cell invasion (324). While the use of collagen I coated transwells for studies of ovarian cancer cell invasion has so far been limited (303), Matrigel-based invasion assays have been used extensively to monitor the invasive potential of a variety of cancer cell types (325), including the role of MT1-MMP in this process using ovarian cancer cells (326). Building on evidence in the latter report that MT1-MMP is an important player in ovarian cancer cell invasion, our experiments provide multiple lines of evidence that GnRH-II-up-regulated MT1-MMP is required for the GnRH-II-enhanced invasion of two human ovarian cancer cells in Matrigel-based invasion assays. However, it will be of interest to assess the role(s) of GnRH-II and its downstream signaling in ovarian cancer cell using both Matrigel and collagen I coated transwells, in order to determine how the GnRH-II treated cancer cells respond to different types of ECM.

In summary, our studies reveal a novel mechanism by which GnRH-II-enhances invasion in ovarian cancer cells. In this scenario (Fig. 5.7), we propose that GnRH-II stimulation of ovarian cancer cell results in the phosphorylation of Akt and induces PI3K/Akt signaling, which subsequently enhances the phosphorylation of GSK3β and activates β-catenin signaling. The translocation of β-catenin into the nucleus up-regulates the β-catenin-target gene, *MT1-MMP*, and thereby contributes to the autocrine actions of GnRH-II in ovarian cancer cell metastasis.
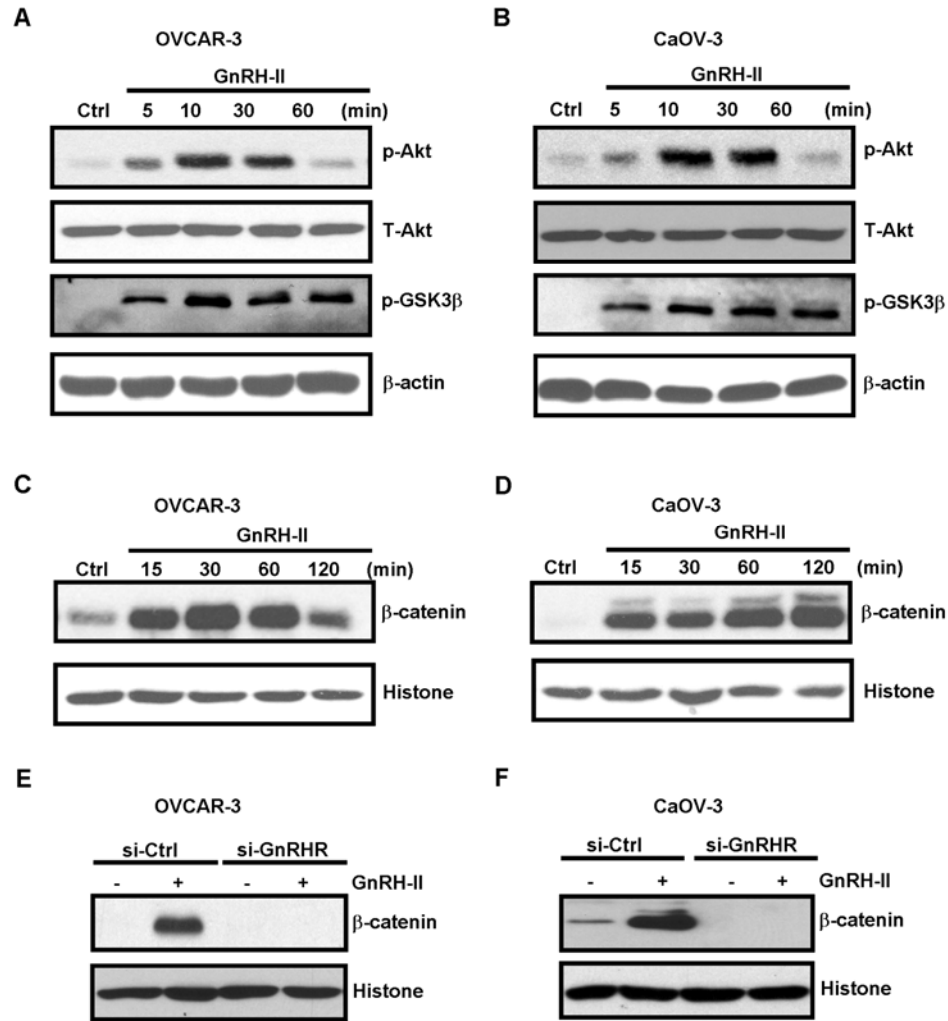
FIG. 5.1. GnRH-II increases the phosphorylation of Akt and GSK3β and induces β-catenin nuclear translocation in ovarian cancer cells. (A) OVCAR-3 and (B) CaOV-3 cells were treated with 10 nM GnRH-II for different times (5, 15, 30, 60 min). Cells harvested were subjected to Western blotting to detect the phosphorylation of Akt (p-Akt) and the phosphorylation of GSK3β (p-GSK3β). Total Akt (T-Akt) and β-actin were used as a normalization control. (C) OVCAR-3 cells and (D) CaOV-3 cells were treated with 10 nM GnRH-II for different times (15, 30, 60, 120 min). Treated cells were then harvested and their nuclear lysates were subjected to Western blotting to measure β-catenin levels. (E) OVCAR-3 and (F) CaOV-3 cells were treated with 100 nM GnRHR siRNA (si-GnRHR) or 100 nM control siRNA (si-Ctrl) for 24 h and then treated with 10 nM GnRH-II for a further 30 min. Treated cells were harvested for the preparation of nuclear cell β-catenin levels were determined by Western blotting. Histone was used as a normalization control in (C-F). Western blots are representative of results from three independent experiments.

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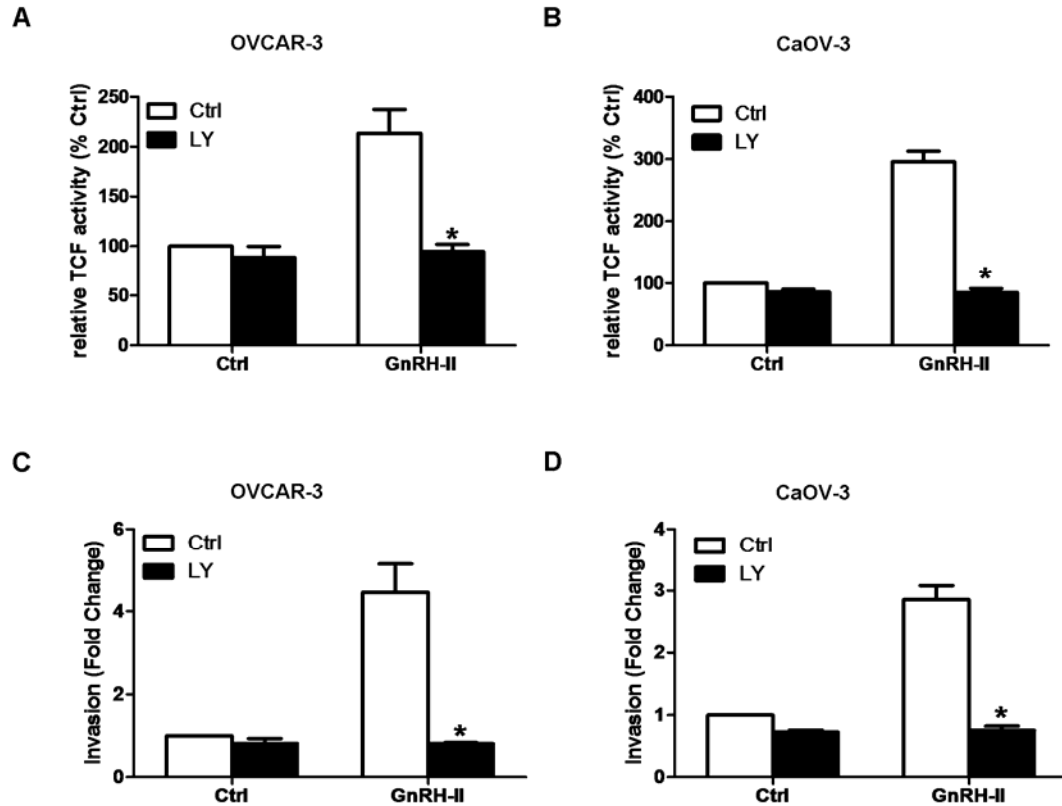
FIG. 5.2. **GnRH-II-induced PI3K/Akt signaling increases β-catenin-dependent luciferase reporter gene activity and promotes ovarian cancer cell invasion.** (A) OVCAR-3 cells and (B) CaOV-3 cells were transfected with a TOPFlash luciferase reporter gene plasmid for 24 h. Transfected cells were then pre-treated with 10 μM LY294002 (LY) for 30 min prior treatment with 10 nM GnRH-II for 24 h. Cell lysates were collected for luciferase assays, and measurements of β-galactosidase activity as a control for transfection efficiency. Results are expressed as mean ± SEM of three independent experiments. *$p<0.05$ compared with untreated control (Ctrl). (C) OVCAR-3 and (D) CaOV-3 cells were pre-treated with 10 μM LY294002 (LY) for 30 min and then seeded into Matrigel-coated transwells and cultured for 48 h in the presence or absence of 10 nM GnRH-II. Non-invading cells were wiped from the upper side of the filter and nuclei of invading cells were stained with Hoechst 33258 and counted. Results are expressed as mean ± SEM of three independent experiments. *$p<0.05$ compared with untreated control (Ctrl).
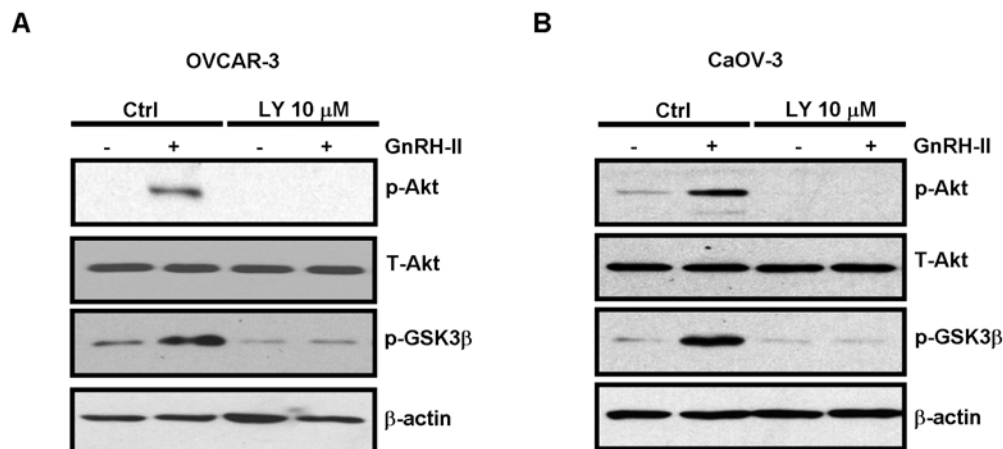
FIG. 5.3. GnRH-II-induced PI3K/Akt signaling increases the phosphorylation of GSK3β. (A) OVCAR-3 and (B) CaOV-3 cells were pre-treated with 10 μM of the PI3K/Akt signaling inhibitor, LY294002 (LY), for 30 min prior treatment with 10 nM GnRH-II for a further 15 min. Treated cells were harvested for Western blotting to detect the phosphorylation of GSK3β (p-GSK3β). Phosphorylated Akt (p-Akt) and total Akt (T-Akt) were probed to determine the efficiency of LY treatments, and β-actin was used as a normalization control. Western blots are representative of results from three independent experiments.
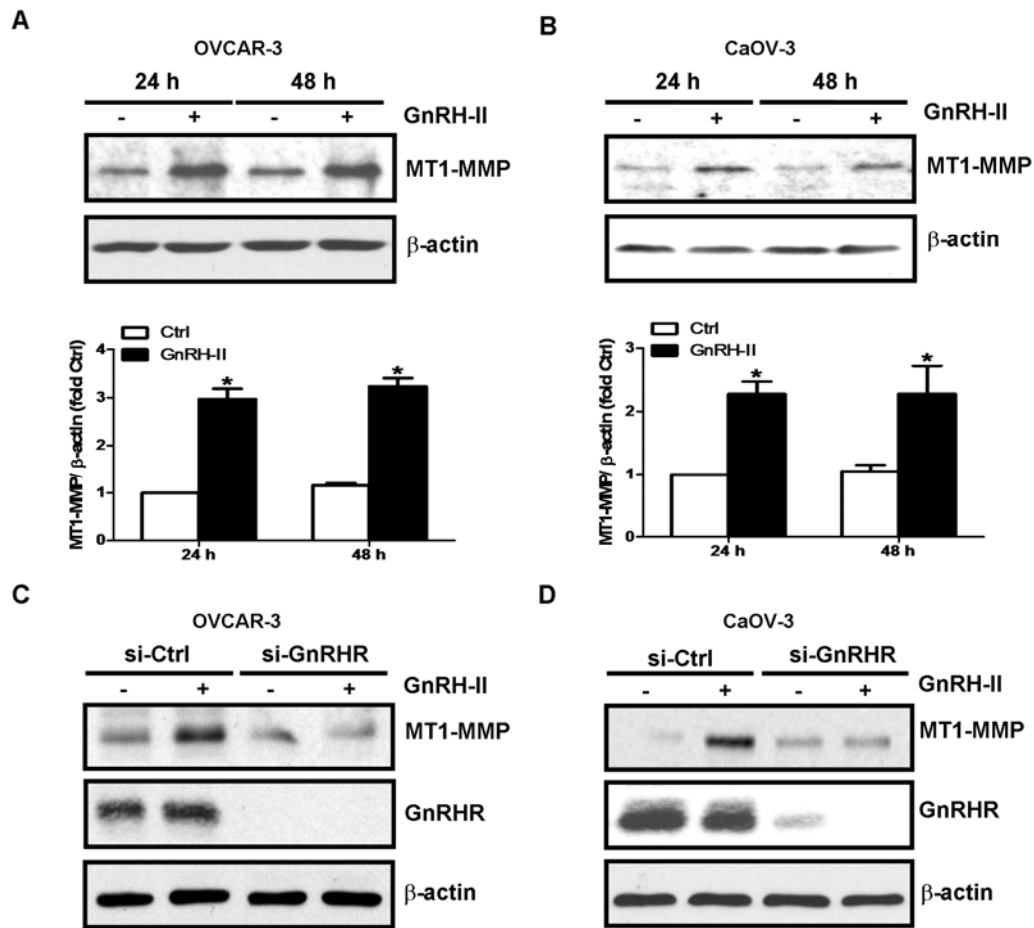
FIG. 5.4. GnRH-II increases MT1-MMP production in ovarian cancer cells. (A) OVCAR-3 and (B) CaOV-3 cells were treated with 10 nM GnRH-II for 24 h or 48 h. Cells harvested were then subjected to Western blotting and probed for MT1-MMP levels. β-actin was used as a normalization control. Upper panel shows a representative Western blot and lower panel shows the quantitative results expressed as mean ± SEM of three independent experiments. *$p<0.05$ compared with untreated control (Ctrl) of the indicated time point. (C) OVCAR-3 cells and (D) CaOV-3 cells were treated with 100 nM GnRHR siRNA (si-GnRHR) or 100 nM control siRNA (si-Ctrl) for 24 h, and then treated with 10 nM GnRH-II for a further 24 h. Treated cells were harvested and cell lysates were prepared to measure MT1-MMP levels. GnRHR was detected to confirm the efficiency of the GnRHR siRNA treatments, and β-actin was used as a normalization control.
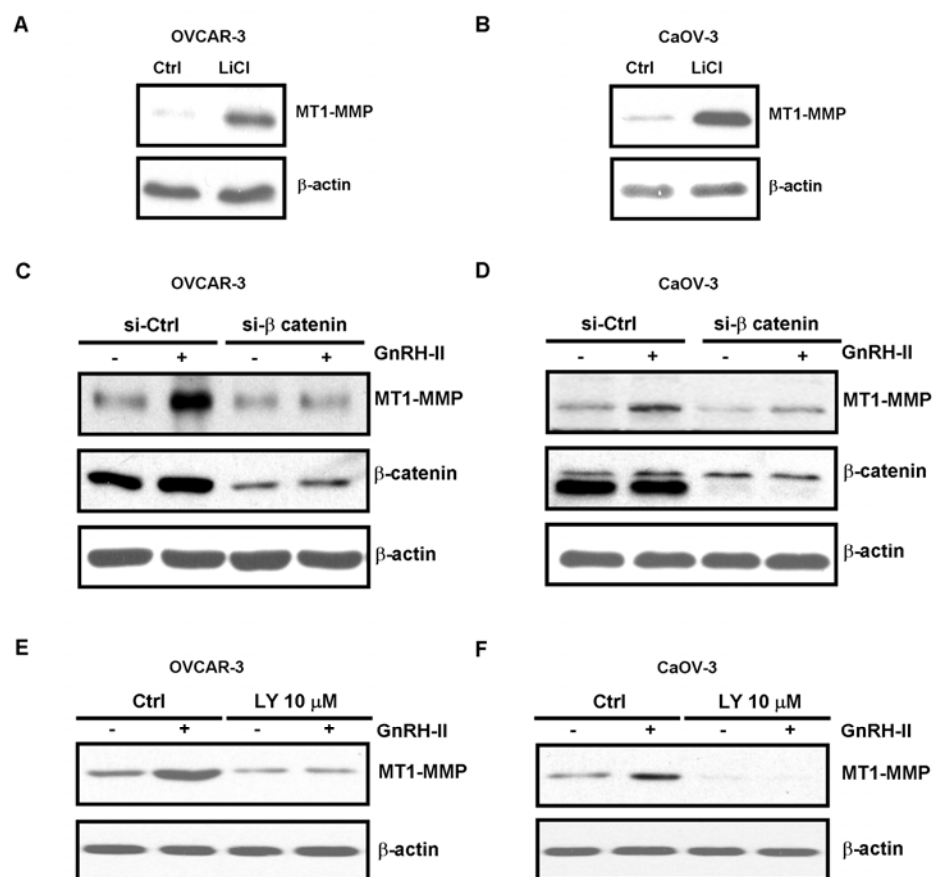
FIG. 5.5. PI3K/Akt and β-catenin signal transduction pathways regulate GnRH-II-induced MT1-MMP production in ovarian cancer cells. (A) OVCAR-3 and (B) CaOV-3 cells were treated with 20 μM LiCl for 24 h. Treated cells were harvested and their cell lysates were subjected to Western blotting to measure MT1-MMP levels. (C) OVCAR-3 cells and (D) CaOV-3 cells were treated with 50 nM β-catenin siRNA (si-β-catenin) or 50 nM control siRNA (si-Ctrl) for 24 h prior to treatment with 10 nM GnRH-II for a further 24 h. Cell lysates were then harvested to measure the protein levels of MT1-MMP by Western blotting. The efficiency of the β-catenin siRNA was verified by Western blotting and β-actin was used as the normalization control. (E) OVCAR-3 and (F) CaOV-3 cells were pre-treated with 10 μM LY294002 (LY) for 30 min prior treatment with 10 nM GnRH-II for a further 24 h. Treated cells were harvested and cell lysates were used to measure MT1-MMP levels by Western blotting and β-actin was used as a normalization control.

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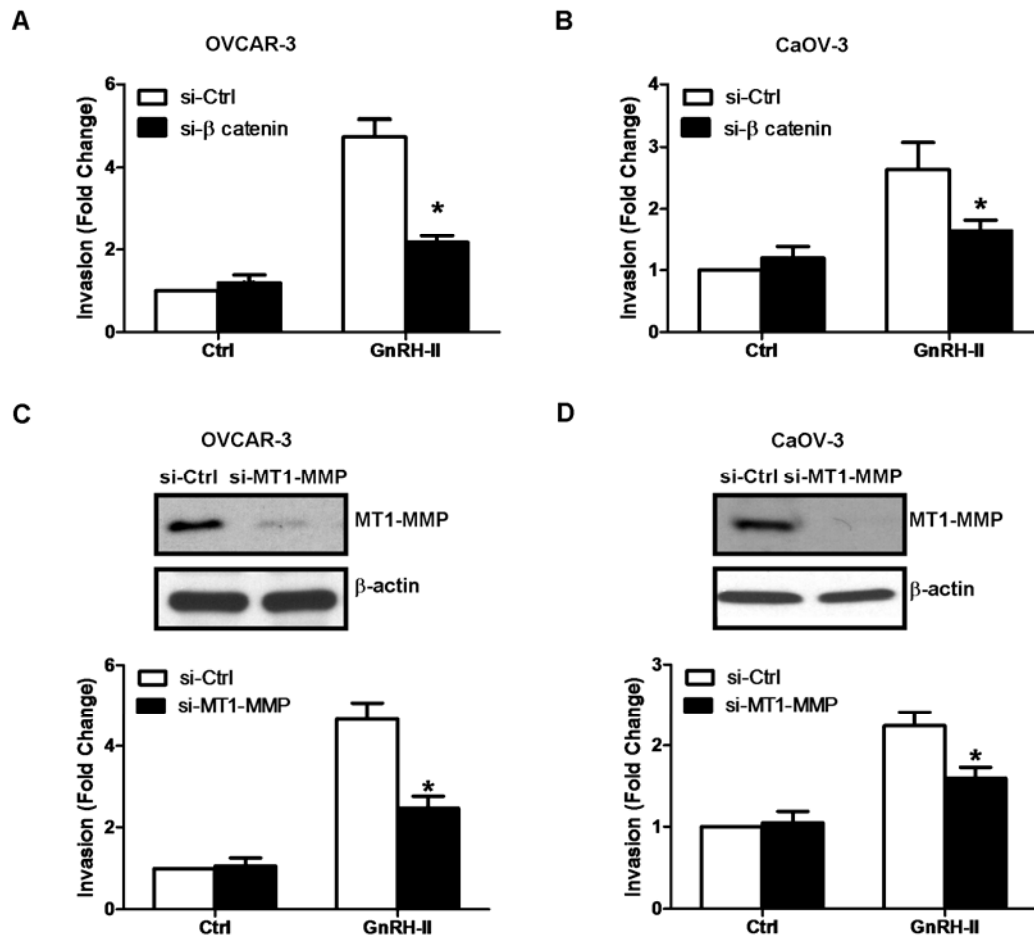
FIG. 5.6. β-Catenin signaling and MT1-MMP contribute to GnRH-II-enhanced ovarian cancer cell invasion. (A) OVCAR-3 and (B) CaOV-3 cells were treated with 50 nM β-catenin siRNA (si-β-catenin) or 50 nM control siRNA (si-Ctrl) for 24 h prior to an invasion assay in the presence or absence of 10 nM GnRN-II for a further 48 h. Results are expressed as mean ± SEM of three independent experiments. *p<0.05 compared with control siRNA (si-Ctrl). (C) OVCAR-3 and (D) CaOV-3 cells were treated with 50 nM MT1-MMP siRNA (si-MT1-MMP) or 50 nM control siRNA (si-Ctrl) for 24 h prior to an invasion assay in the presence or absence of 10 nM GnRH-II for a further 48 h. The efficiency for MT1-MMP siRNA was verified by Western blotting. Results are expressed as mean ± SEM of three independent experiments. * p<0.05 compared with control siRNA (si-Ctrl).
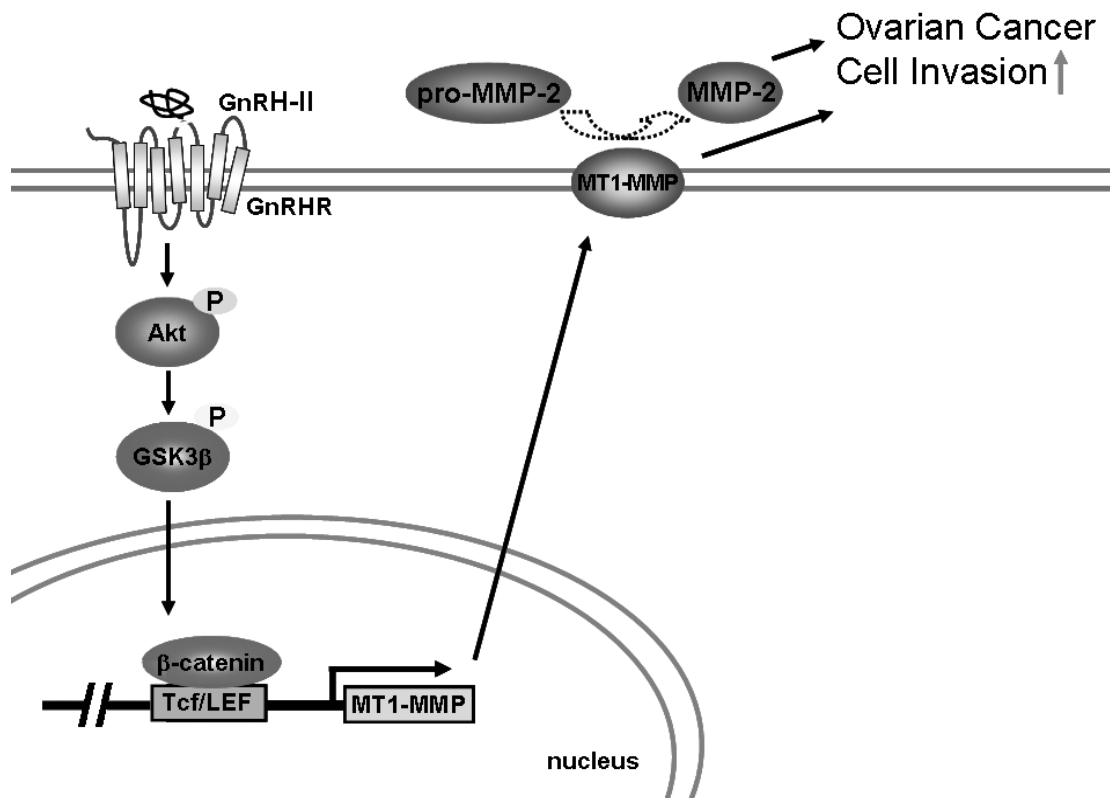
FIG. 5.7. Proposed model illustrating how GnRH-II induced MT1-MMP may contribute to ovarian cancer cell invasion. GnRH-II acts *via* the GnRHR to induce the phosphorylation of Akt. Phosphorylated Akt stimulates the phosphorylation of GSK3β and this thereby releases β-catenin, which can then and translocate to the nucleus. The accumulation of β-catenin in the nucleus acts on the TCF/LEF site within the *MT1-MMP* promoter which causes an up-regulation of *MT1-MMP* expression in ovarian cancer cells. Increased production of MT1-MMP contributes to the GnRH-II-enhanced ovarian cancer cell invasion by increasing the cleavage of proMMP-2 zymogen into its active form. GnRHR, GnRH receptor; p-Akt, phosphorylated Akt; p-GSK3β, phosphorylated glycogen synthase 3β; TCF/LEF, T cell factor/lymphoid enhancer binding factor; MT1-MMP, membrane type I matrix metalloproteinase; MMP-2, matrix metalloproteinase-2.

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CHATER 6. Expression of the 67 kDa non-integrin laminin receptor in high-grade serous ovarian carcinoma

6.1 Introduction

Ovarian cancer is the most lethal gynecological malignancy and the fourth most common cause of female cancer deaths (100) (Canadian Cancer Society Stats). Each year, there are approximately 42,000 new cases in Europe (101) and 22,000 new cases in the USA (102). Approximately 90% of ovarian cancers are epithelial carcinomas (327) and these are classified by their histological features. Most common are serous (50%) tumor followed by endometrioid (20%), mucinous (10%), clear cell (5%), and also some rare cases including transitional, mixed, or undifferentiated (<5%) (328-330). Mortality and morbidity from this cancer arise mainly from intra-abdominal disease spread (205). Thus, it is important to elucidate the biology of how ovarian cancers metastasize. This knowledge could lead to the development of new prognostic tools and, more importantly, to better disease treatment.

The degradation and invasion of the basement membrane (BM) by malignant cells is a crucial step in the local invasion and distant metastasis of aggressive epithelial malignancies (331-333). It involves multiple steps, including cell adhesion, extracellular matrix degradation, and subsequent migration of cancer cells (334). Several gene products have been identified as key elements in BM penetration, including various cell surface laminin receptors. Among these, the 67 kDa high-affinity non-integrin laminin receptor (67LR), which was first isolated from cancer cell membranes (173-175), exhibits increased expression in many highly metastatic cancer cells (177). 67LR expression is also associated with poor differentiation, disease progression and poor survival (180, 281). Studies demonstrated that the mature 67LR is formed by the homodimerization of a 37 kDa laminin receptor precursor protein (LRP) (180, 181). Thus, LRP levels can be used as an index to reflect the levels of 67LR on the cell surface.

Clinically, LRP levels have been correlated with the presence of malignancy (205), high histological grade (206), suboptimal debulking (205) and poor outcome (205, 206) in ovarian carcinoma (207). In line with these studies, our group demonstrated in an *in vitro* model that altered LRP levels correlate with the invasiveness of ovarian cancer cell lines and the up-regulation of pro-invasive target gene expression (Chapter IV). Although there is some controversy in the literature, we aimed to clarify the role of LRP or 67LR in ovarian carcinoma. We analyzed LRP levels in a well-studied population of ovarian cancer patients using tissue microarray, with the goal of determining whether this protein is correlated with clinical outcomes and survival, particularly in high-grade serous ovarian carcinoma.

6.2 Materials and methods

Patient population

Approval for the study was obtained from the ethics committee of the University of British Columbia. Most women diagnosed with ovarian cancer in British Columbia are treated at the British Columbia Cancer agency (BCCA) and provincial treatment guidelines were followed. Outcomes are tracked via the Cheryl Brown Ovarian Cancer Outcomes Unit, an ovarian cancer database of the BCCA. 445 cases, dated between the years of 1994 – 2004, were considered for tissue microarray (335). Due to insufficient sample size, we excluded patients diagnosed with mucinous, clear cell, endometroid, and low-grade serous ovarian carcinoma (Table 6.1). In order to avoid potential confounding clinical variables we restricted our analysis to high-grade serous cancers of advanced stage (III and IV) all of whom were treated by initial surgery. We excluded patients treated by neoadjuvant chemotherapy and those that were histologically diagnosed as non-epithelial cell tumors or metastatic tumors (Table 6.2).

Tissue microarray (TMA) and immunohistochemistry

The ovarian cancer TMA construction was as previously described (335). A gynecological pathologist performed a blinded full-slide review of the 632 cases. Tumor cell type and grade (Silverberg) were assessed. After review, 445 cases of invasive ovarian carcinoma were available in tissue blocks for tissue microarray construction. A representative area of each tumor was selected, and a duplicate core TMA was constructed (Beecher Instruments, Silver Springs, MD, USA). Serial 4 μm sections were cut for immunohistochemical (IHC) analysis and run through an automated protocol, including heat antigen retrieval (Ventana System) (335). LRP monoclonal antibody (MLuC5; Santa Cruz, Cat# SC59732) was optimized prior usage.

We scored both membrane and vascular staining, and did not assess cytoplasmic staining. 67LR staining was scored as 0 for negative cases, +1 for mild staining (~1% to 5%), and +2 for intense staining (>50%) (Fig. 6.1A, B, C). Vascular staining of 67LR was graded as present or absent (Fig. 6.1D). If present, there had to be obvious and uniform staining of most of the vessels in the core examined.

Statistical analysis

Univariable survival analysis was performed by the generation of Kaplan-Meier curves, and differences between groups were assessed using the logrank statistic (335). Multivariable survival analysis was performed using the Cox Proportional Hazards Model (335). Contingency tables and the Pearson chi-square statistic were used to assess changes in the distribution of LRP expression across different stages of high grade serous ovarian carcinoma (335). All analyses were performed using JMP version 6.0.3 (SAS Institute, Cary NC, USA).

6.3 Results

Expression of the 67LR in ovarian carcinoma samples

Of the 120 cases of high-grade serous carcinoma (stage III & IV), 19 cases (16%) scored +2, 25 cases (21%) scored +1 and 76 cases (63%) were negative for 67LR. Our contingency analysis showed that cancer stage (III & IV) correlates with the membrane staining of 67LR (p=0.0202). The Kaplan-Meier analysis showed that the expression of 67LR does not correlate with the overall survival of these cases (Fig. 6.2).

Of the same 120 cases high-grade serous ovarian carcinoma (stage III & IV), 101 cases (84%) were negative for 67LR staining in the vessels while 18 cases (15%) scored positive. Contingency analysis showed that stage (III & IV) does not correlate with 67LR positivity in vessels (p=0.2968) and Kaplan-Meier analysis showed that the expression of 67LR in vessels does not correlate with the overall survival of patients (Fig 6.3). However, when all stages (I, II, III, IV) of high-grade serous ovarian carcinoma were considered, Cox regression analysis showed that vessel staining conferred a better outcome, though this difference was of borderline statistical significance (p=0.0425).

6.4 Discussion

The expression of 67LR has been correlated with the prognosis of a variety of carcinomas (281). Therefore, it has been suggested that the detection of 67LR in tumor cells may constitute the basis for new strategies of evaluating prognosis. Presently, our tissue microaaray results show that the membrane staining of 67LR correlates with stages in high-grade serous ovarian carcinoma (stage III & IV). However, the expression of 67LR does not correlate with the overall survival of these patients. Instead, the presence of 67LR within the vessels of tumors may confer a better outcome for these patients.

Studies of the prognostic role of 67LR in ovarian carcinomas have been inconclusive. Castronovo *et al.* reported that ovarian cancer patients who experience subsequent progression of the disease (i.e., alive with recurrence or dead of the disease) and patients with high-grade tumors were characterized by significantly higher levels of 67LR membrane staining in their tumor specimens (205, 206). Reich *et al.* reported that the high expression of 67LR in tumor cells did not predict disease outcome in either effusions or solid tumors (207). These results may be confounding because the cohort of patients used by Castronovo *et al.* consisted of different histotypes (i.e., serous, mucinous, endometrioid, clear cell and undifferentiated) and stages (stages I, II, III and IV) of ovarian carcinoma, whereas the patients used in the Reich study included both primary and metastatic lesions of serous ovarian carcinoma. Moreover, the populations in the above studies also included patients who were treated with chemotherapy, which may also contribute to the contradictory results in terms of patient outcomes. Because the etiologies of different ovarian carcinoma histotypes (i.e., low-grade serous, high-grade serous, mucinous, endometrioid, clear cell and undifferentiated) are distinct and the biological aggressiveness and behavior of each subtype is variable (336), our present study only focused on patients with high-grade stage III & IV serous ovarian carcinoma, one of the most common and lethal subtypes of ovarian cancer. We excluded those patients who had received neoadjuvant therapy. In this regard, our results showed that although membrane staining of 67LR is correlated with stage in high-grade serous ovarian carcinoma, it does not have prognostic value in this subtype of ovarian carcinoma.

The basement membrane is comprised of different components in a location-specific manner. In this regard, 67LR may associate with different laminin subunits, integrin subtypes or other membrane-bound proteins, leading to distinct effects in a cell type-specific manner. This assumption was evident in *in vitro* and *ex vivo* studies, which have demonstrated that 67LR acts as a receptor for the green tea polyphenol epigallocatechin-3-gallate (337-339) and pigment

epithelium-derived growth factor to induce apoptosis or inhibit endothelial cell growth (340). These results contradict its role in promoting tumor progression. While the over-expression of 67LR within the vascular endothelium endothelial cells of a variety of tumor types has been well documented (341), both Castronovo and Reiche stated that 67LR staining occurred in the vasculature of their patients. However, they did not clarify whether this staining was histotype-specific nor did they provide statistical data on the significance of 67LR expression in the vessels of ovarian carcinomas. Presently, we have now found that although expression of 67LR within the vessels of stage III & IV high-grade serous ovarian carcinoma did not have any prognostic value, analysis including all stages of high-grade serous patients showed that this 67LR vessel staining confer better outcomes.

The interactions between tumor and stromal cells are now considered to play a significant role during tumor development and progression. For example, our present results agree with our *in vitro* studies in that 67LR is expressed in highly invasive ovarian carcinoma. Intriguingly, 67LR expression within the vasculature may have contradictory effects compared to its role in tumor cells. This apparent contradiction is interesting and leads to a series of questions: what role might 67LR play in endothelial cell functions, and, specifically, how might this receptor affect ovarian cancer metastasis? Larger prospective studies are needed to validate the prognostic role of 67LR vessel staining in ovarian cancer patients, particularly those patients with high-grade serous ovarian carcinoma.

Histological subtypes	67 LR membrane positive (%)	Cases
Clear cell	11.5 %	26
Mucinous	14.2 %	7
Endometrioid	42.8 %	28
Low grade serous	50 %	6
High grade serous	39.3%	150

Table 6.1. Percentage of 67LR membrane staining positive cases within the histological subtypes.
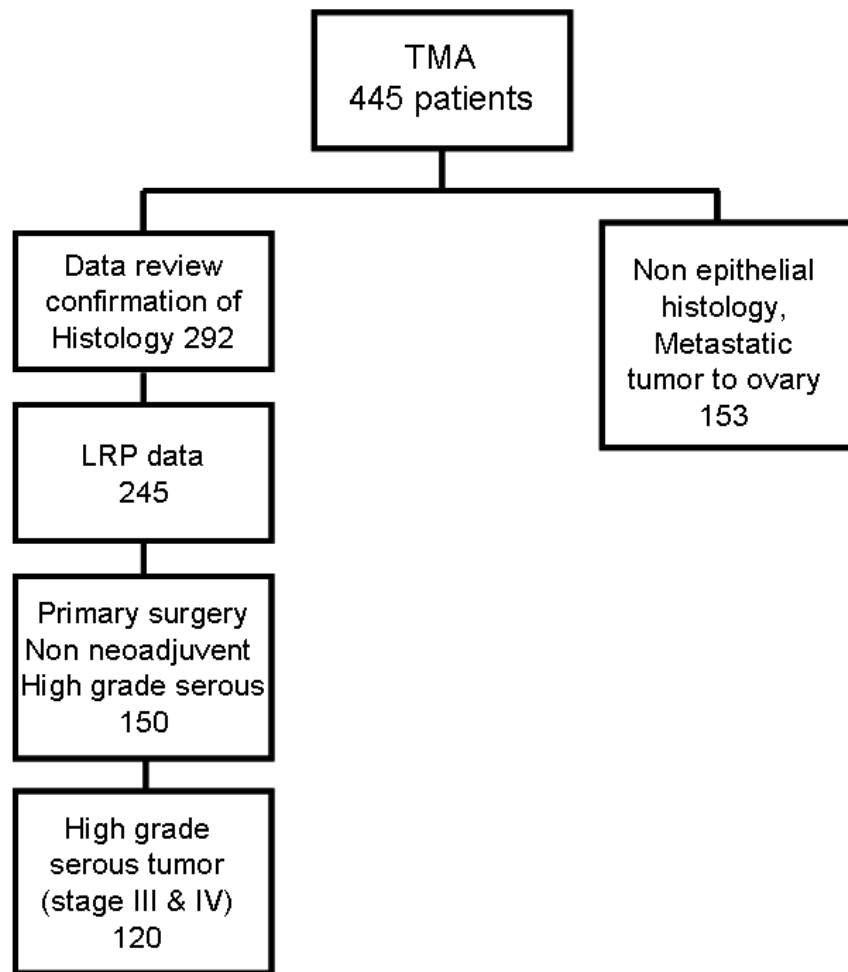
Table 6.2. Study population in the present analysis.
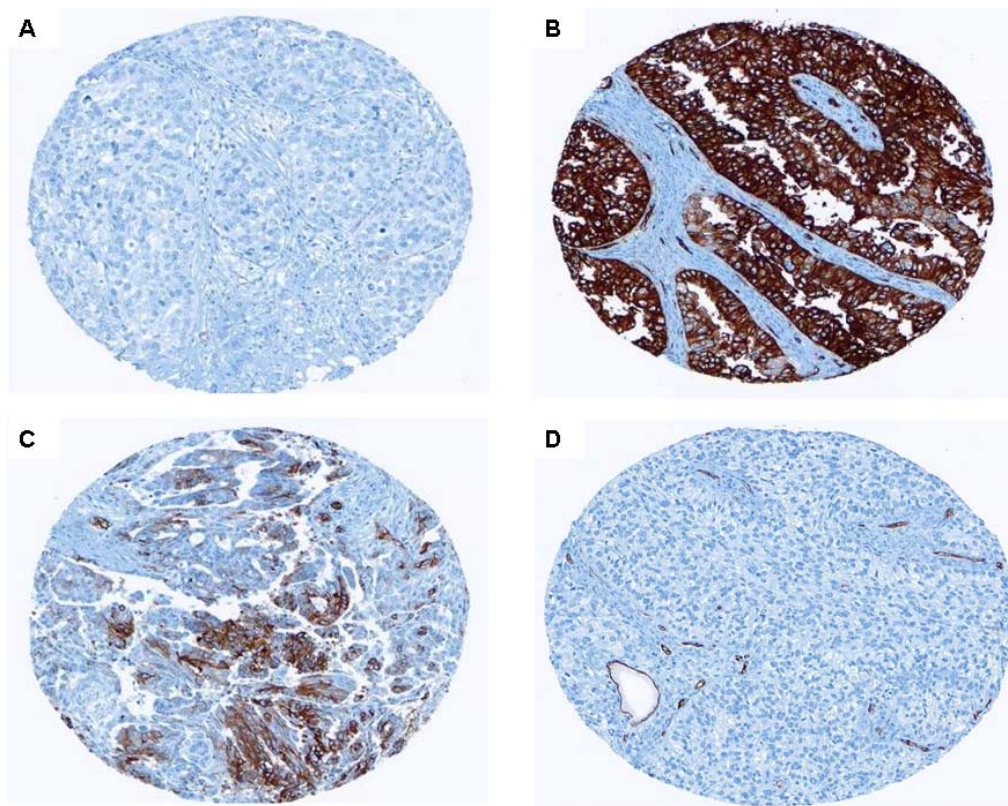
FIG. 6.1. Immunoreacitivty pattern of LRP. (A) Representative sample of negative cases show complete lack of staining and are classified as 0. (B) Represntative sample of +2 intense immunoreacitivy exhibits dark brown staining in tumor cell membrane. (C) Representative sample of +1 intense immunoreactivity exhibits moderate brown staining in tumor cell membrane. (D) Representative sample showing LRP staining in vessels.
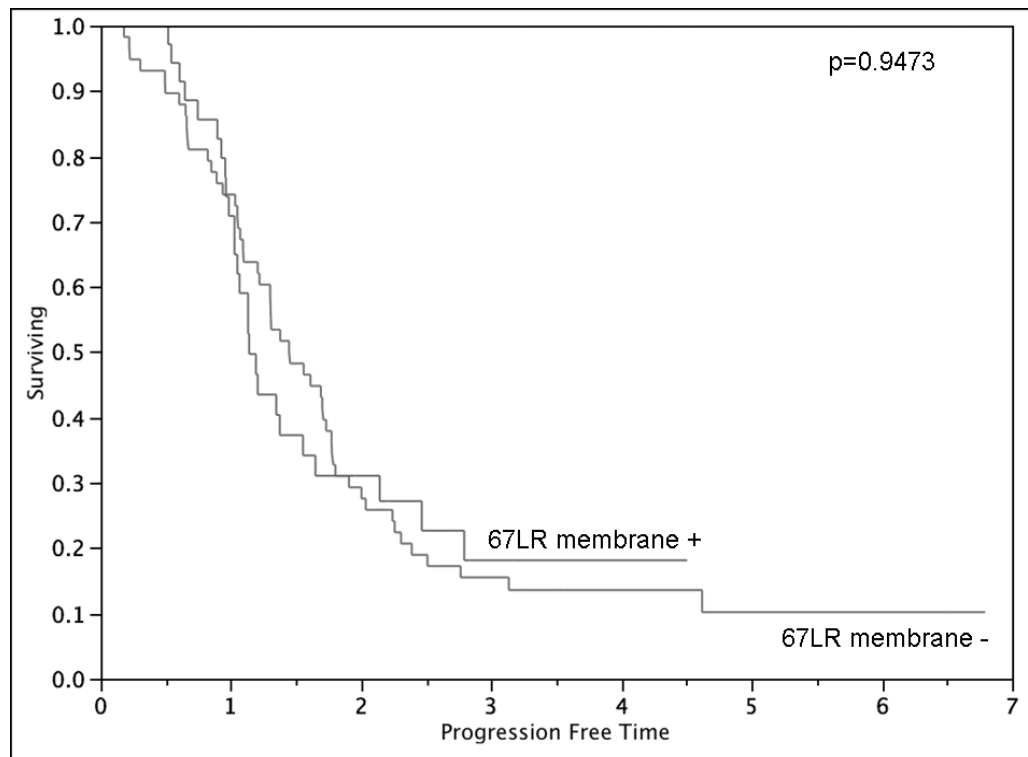
FIG. 6.2. Kaplan-Meier overall survival curve for 67LR membrane staining in stage III and IV high-grade serous ovarian carcinoma.
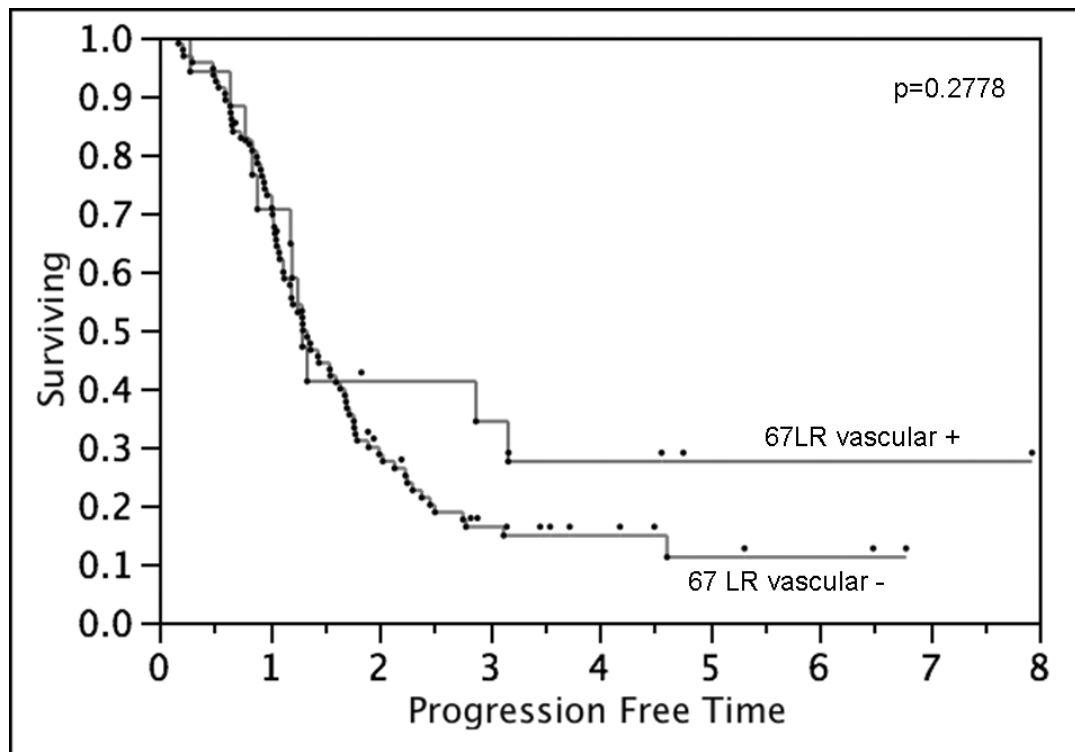
FIG. 6.3. Kaplan-Meier overall survival curve for 67LR vessel staining in stage III and IV high-grade serous ovarian carcinoma.

CHAPTER 7. Conclusion and future directions

7.1 Overview

GnRH-II has been proposed to act in an autocrine/paracrine manner during cancer progression. However, studies designed to test this hypothesis have been limited to the preliminary characterization of *GnRH-II* expression, and they have not addressed in detail how GnRH-II functions as an autocrine regulator in cancer cell biology. Thus, the studies described herein were designed to delineate the molecular regulation of *GnRH-II* in ovarian cancer cell lines and to verify the role of GnRH-II and its downstream effectors in these cells.

First, we demonstrated that the cAMP-responsive element is a *cis*-acting element that is necessary and sufficient for both basal (Chapter II) and EGF-induced (Chapter III) human *GnRH-II* promoter activity. We then verified that EGF-induced GnRH-II production enhances the invasive potential of ovarian cancer cells (Chapter III). We built upon these results in the subsequent chapters, which describe the downstream mechanisms of GnRH-II/GnRHR autocrine action in ovarian cancer cells (Chapter IV and Chapter V).

Ovarian cancer metastasis is a complex process that involves the detachment of tumor cells from the primary lesion and the subsequent attachment of these cells to the peritoneum or omentum (342, 343). In this regard, increased expression of matrix metalloproteinases (MMPs) (143) and/or adhesion molecules (344) may favor the remodeling of the extracellular matrix, thereby resulting in the degradation of the basement membrane, which would allow the tumor cells to invade surrounding tissue. We have found that GnRH-II/GnRHR autocrine actions stimulate the expression of MMP-2 (Chapter IV) and MT1-MMP (Chapter V) in ovarian cancer cells. In this context, the increased production of the 67 kDa non-integrin laminin receptor (67LR) induced by GnRH-II is a critical intermediary step, which leads to an interaction between the 67LR and laminin and the induction of MMP-2 expression in ovarian cancer cells

(Chapter IV). The increased expression of MT1-MMP is regulated by β-catenin signaling, which is activated by GnRH-II-induced PI3K/Akt signaling (Chapter V). Taken together, the present studies represent a model system that provides insight into the regulation of *GnRH-II* expression and its potential functional role in ovarian cancer carcinoma (Fig. 7.1).

7.2 The importance of EGF-induced GnRH-II production

Compelling evidence suggests that hormones and growth factors influence the occurrence of ovarian carcinoma (71, 345). Among the variety of growth factors and cytokines tested in our cell model (i.e., EGF, HGF, FGF, leptin; Appendix I), only EGF was found to rapidly stimulate *GnRH-II* transcription. Only GnRH-II, not GnRH-I, is regulated by EGF/EGFR (Chapter III). This result may be explained by the distinct *cis*-acting elements that are required for the regulation of *GnRH-I* and *GnRH-II* transcription. In this regard, the *GnRH-I* promoter region lacks the cAMP-responsive element (11), which plays a critical role in driving basal *GnRH-II* transcriptional activity.

Interestingly, we found that EGF regulates *GnRH-II* transcriptional activity in human choriocarcinoma JEG-3 cells (Appendix II) in a manner similar to ovarian cancer cell lines. Moreover, this increase in GnRH-II production contributes to the invasive potential of JEG-3 cells (Appendix II). Elevated levels of GnRH-II and GnRHR have been reported in placental tissues during the first trimester of pregnancy (68, 346). In line with these reports, studies have demonstrated that GnRH-II treatment enhances the expression of matrix metalloproteinases and promotes extravillous trophoblast cell invasiveness (68, 292). Taken together, we propose that cross talk between EGF/EGFR and GnRH-II/GnRHR signaling pathways may constitute an autocrine regulatory system that promotes implantation, and it deserves further investigation.

7.3 The role of LRP in ovarian cancer cell invasive potential

In chapter V, we observe that the ranking of LRP levels in the three ovarian cancer cell lines (SKOV-3>CaOV-3>OVCAR-3) corresponds to their invasiveness (i.e., from most invasive to least invasive). However, it should be noted that SKOV-3 cells have only limited GnRHR levels, and consequently did not respond to GnRH-II treatment. In other words, the regulation of *LRP* expression in this highly invasive cell line is independent of the autocrine action of GnRH-II/GnRHR. Thus, further studies are required to fully understand the complex regulation of *LRP* expression in ovarian cancer cells.

Several LRP modulated target genes were identified using our *in vitro* model. Over-expression of LRP induces N-cadherin expression in ovarian cancer cells (Appendix III). Cadherin switching occurs during epithelial-mesenchymal transition and thereby plays a critical role during tumor progression (347). Although the expression of E-cadherin was not affected in these LRP over-expressing cells, we also know that increased N-cadherin is required for the LRP-mediated increase in ovarian cancer cell invasiveness (Appendix III). These results are consistent with past studies in which both laminin and integrin signaling mediate the expression of N-cadherin during tumor progression (348). They do, however, imply that the interaction between 67LR and laminin regulates the expression of distinct molecules that are important during ovarian tumorigenesis (202, 349, 350). It will therefore be worthwhile to investigate the role of LRP in ovarian cancer cell progression in greater detail.

Although our *in vitro* findings suggest that 67LR plays a key role in determining the invasive potential of ovarian cancer cells, our tissue microarray studies indicate that the expression of 67LR does not correlate with the overall survival of high-grade serous ovarian carcinoma patients (stage III & IV). Nevertheless, we did observe a trend in the expression of 67LR in other ovarian carcinoma subtypes, but a statistical analysis with such a limited sample size of other subtypes would not be conclusive. We are therefore now performing another tissue

microarray analysis with a larger number of patient samples to assess the role of 67LR in ovarian carcinoma.

7.4 The role of GnRH-II/GnRHR-induced MMP-2 and MT1-MMP in ovarian cancer cells

MMP-2 (268), -9 (166), and MT1-MMP (304, 351) are among the most studied MMPs in ovarian carcinoma. The expression of these three proteases increases in advanced ovarian cancers (International Federation of Gynecology and Obstetrics stage III) more so than in benign tumors. Increased expression of each proteinase was found to be an independent prognosticator of poor survival or cancer progression (164, 352, 353). Not only do the tumor cells secrete these enzymes, but the stromal cells around the tumor may also contribute to the up-regulation of these enzymes during tumor-stromal cell interactions, thus contributing to cancer progression (144).

In our cell system, we found that GnRH-II up-regulates the production of both MMP-2 and MT1-MMP in a similar time frame (Chapter IV and V). However, the downstream signaling events involved in the regulation of these two enzymes are distinct and independent of one another (Chapter IV, V and Appendix). Upon comparing the promoter regions of *MMP-2* and *MT1-MMP*, only *MT1-MMP* possesses an intact β-catenin recognition motif (TCF/LEF binding site) and is known to respond to the stimulation of Wnt/β-catenin signaling (305, 354, 355). Studies of *MMP-2* demonstrated that there are no TCF binding sites within its promoter region. Instead, intact AP-1 (24, 356) and SP-1 (357) recognition motifs were shown to be important for *MMP-2* transcriptional activity (358). In fact, both AP-1 and SP-1 transcription factors are known to be regulated by GnRH/GnRHR downstream signaling (2, 53). Thus, it would be worthwhile to study the impact of GnRH-II/GnRHR signaling on *MMP-2* and *MT1-MMP* promoter activity.

Studies have shown that MT1-MMP may itself degrade the ECM (359) or may act as a physiological activator of pro-MMP-2 (158, 360). In either case, these effects promote cancer cell invasion (297). Our present studies demonstrated that both proteinases contribute to ovarian cancer cell invasiveness (Chapter IV and V). However, siRNA-mediated depletion of either MT1-MMP or its signaling regulator, β-catenin, only resulted in partial inhibition of GnRH-II-induced ovarian cancer cell invasion (Chapter V) compared with siRNA-mediated MMP-2 depletion, which led to a total inhibition of GnRH-II-enhanced invasiveness in the same cell lines (Chapter IV). These findings imply that the GnRH-II-induced increases in MMP-2 and MT1-MMP expression may act synergistically to enhance ovarian cancer cell invasiveness. Thus, it would be of interest to verify whether increased MT1-MMP enhances MMP-2 proteolytic activity in our model system. These results may further define the role of these two proteinases in GnRH-II-regulated ovarian cancer cell progression.

Activation of MMP-2 at the cell surface is a complex and unique multistep pathway that involves not only MT1-MMP but also the tissue inhibitor of MMP-2, TIMP-2 (361). TIMP-2 can directly bind with either pro-MMP-2 or MT1-MMP. In this regards, the activated MT1-MMP binds with TIMP-2 and thus allow the cleavage of pro-MMP-2 by MT1-MMP (362). MT1-MMP alone does not fully activate MMP-2; an additional activated MMP-2 is required to remove a residual portion of the MMP-2 propeptide (362) for the full activation of this protease. Thus, the ratio of MMP-2:MT1-MMP:TIMP-2 has been shown to play an important role in the activation of MMP-2 and thereby to enhance cancer cell progression (360). In fact, others have demonstrated a regulatory role of GnRH/GnRHR on TIMP-2 expression (71, 363). Thus, determining the role of TIMP-2 in our model system is worth further investigation.

7.5 The role of GnRH-II/GnRHR in ovarian cancer progression

The initial rationale for the use of GnRH agonists and antagonists as therapeutic drugs in sex steroid-dependent tumors was based on their down-regulation of pituitary GnRHR and the consequent inhibition of gonadotropin and sex steroid secretion (364). The discovery of GnRH subtypes and GnRHR expression in the localized tumor tissues (53) prompted researchers to define the functional role of GnRH in cancer cell biology.

Over the past two decades, *in vitro* studies have demonstrated that the GnRH/GnRHR system exerts anti-proliferative effects in a variety of tumor cell lines (53). Based on these studies, clinical trials explored the potential of GnRH agonists as an adjuvant therapy in a wide range of malignant tumors, including prostate cancer, breast cancer, endometrial cancer and ovarian cancer (364). Although some of the clinical trials (e.g., prostate cancer and endometrial cancer) have shown potential benefits of GnRH agonists (364), results from the phase II clinical trial for ovarian cancer did not show positive effects (95). Thus, our findings and those of others (71, 78, 365), which have shown the pro-invasive effects of GnRH-I and GnRH-II agonists in ovarian cancer, may be important for future evaluations of GnRH agonist clinical trials.

The contradictory finding that GnRH agonists can have both anti-proliferative and pro-invasive effects can be explanined. A similar discrepancy has been observed for the PI3K/Akt signaling pathway (366). Akt is well studied for its role as a pro-survival molecule that increases the proliferation of a variety of tumor cells (366). However, compelling evidence indicates that Akt signaling inhibits the invasiveness of tumor cells in a cell-type-specific manner (366). One possible explanation for the contradictions in both the GnRH/GnRHR system and Akt signaling may be attributed to the distinct and complex downstream molecules that are present in differential cell types. In addition, the diversity of downstream signaling pathways, which are initiated upon GnRHR activation, may be attributed to the differential functional roles of GnRH (52, 53). To further define the role of GnRH/GnRHR in ovarian cancer cell tumorigenesis, *in*

vivo studies in a GnRH-I, GnRH-II, or GnRHR knockout xenograft mouse model would be helpful for monitoring the function of GnRH in this disease.

Recent morphological and molecular genetic studies have raised the question of whether epithelial ovarian carcinomas are all derived from the same origin (105, 367). This notion challenges previous reports that have identified ovarian cancer-associated genes that were "over-expressed" relative to their "normal" counterpart tissue. For example, high-grade ovarian carcinoma is now hypothesized to develop from fallopian tube epithelial cells and has been shown to possess a distinct molecular genetic signature and distinct behavior when compared with other histotypes of ovarian carcinoma (i.e., mucinous, clear cell, endometrioid) (367, 368). In this regard, studies that showed the "high expression" of GnRH subtypes and GnRHR in ovarian cancer were mostly performed by comparing "malignant" to "benign" and/or "normal" ovary tissues (34, 49, 139). Thus, the significance of these studies may have to be readdressed. Furthermore, differences in the etiologies of these ovarian carcinoma subtypes may also result in differences in the expression of different signaling molecules, receptors and tumor suppressor genes (97, 369). The cell lines we used were established in the 1980s; they were not defined as representative of a particular subtype. Thus, developing and using well-defined ovarian cancer subtype cell lines in the future can improve the interpretation of experimental outcomes.

The thesis research contributes to understanding of the impact of GnRH-II/GnRHR on the invasive potential of ovarian cancer, provides insights into the progression of ovarian cancer, and provides new approaches for the evaluation of new therapeutic strategies.
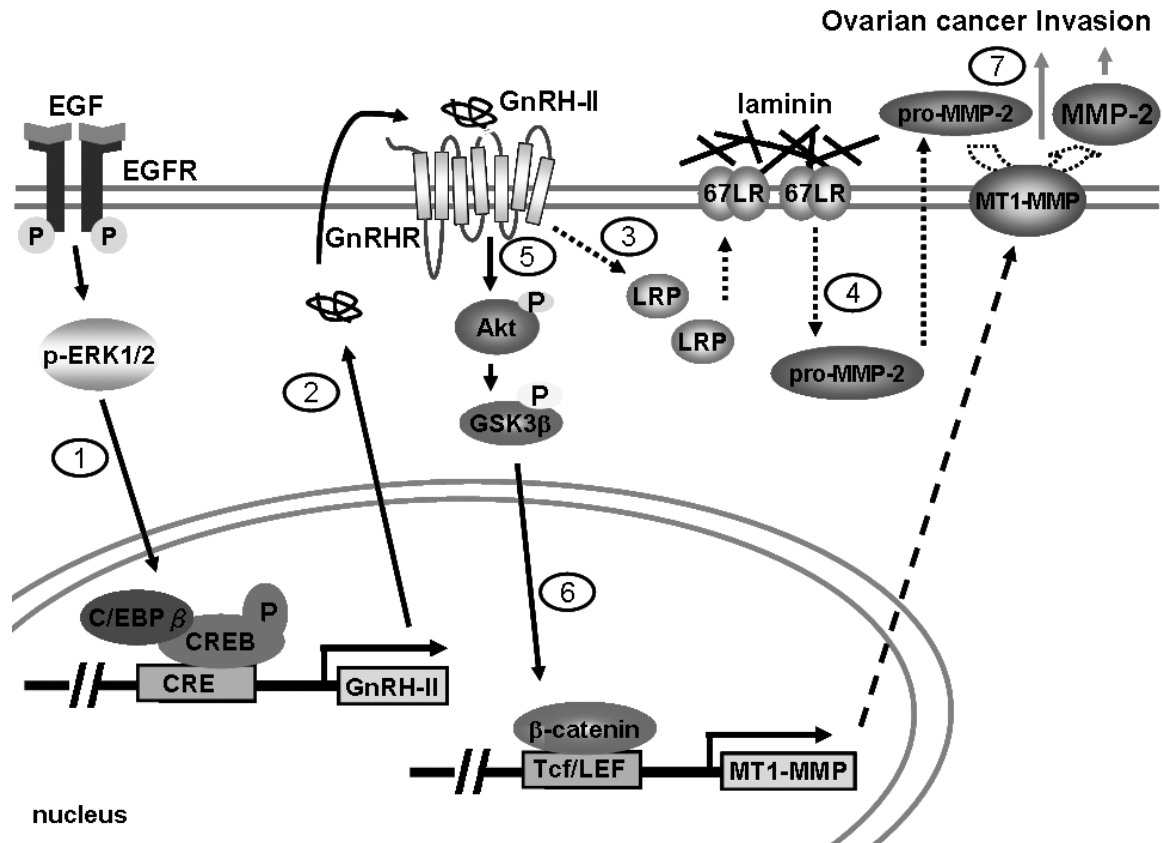
FIG. 7.1. Proposed model for GnRH-II autocrine actions in ovarian cancer cell invasion. (1) Binding of EGF with EGFR induces receptor autophosphorylation, thereby stimulating the phosphorylation of ERK1/2. Phosphorylated ERK1/2 translocates into the nucleus and induces the phosphorylation of CREB and the interaction of C/EBPβ with p-CREB. These transcription factors bind onto the CRE site within the *GnRH-II* promoter and up-regulate its transcription, which increase GnRH-II production. (2) Increased production of GnRH-II acts in an autocrine manner through the GnRHR, and initiate two distinct signaling events. (3) It increases 37 kDa LRP expression which results in increasing surface 67LR in ovarian cancer cells (4), thereby increases the interaction of cancer cells with laminin in the extracellular matrix and this leads to the up-regulation of MMP-2 production. (5) In addition, GnRH-II/GnRHR interactions induce phosphorylation of Akt, and this activates PI3K/Akt signaling which induces GSK3β phosphorylation that promotes (6) the translocation of β-catenin into the nucleus where it stimulates the production of MT1-MMP. (7) Together, these events enhance MMP-2 production and activation which promotes ovarian cancer cell invasion. EGFR, EGF receptor; GnRHR, GnRH receptor; p-ERK1/2, phosphorylated ERK1/2; p-CREB, phosphorylated CREB; C/EBPβ, CCAAT/enhancer binding proteinβ; CRE, cAMP responsive element; LRP, 37 kDa laminin receptor precursor; 67LR, 67 kDa non-integrin laminin receptor; MMP-2, matrix metalloproteinase-2; p-Akt, phophorylated Akt; p-GSK3β, phosphorylated gylcogen synthase 3β; Tcf/LEF, T cell factor/lymphoid enhancer binding factor; MT1-MMP, membrane type I metalloproteinase.

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Appendices.

Appendix I. The effects of growth factors on human *GnRH-II* promoter activity.
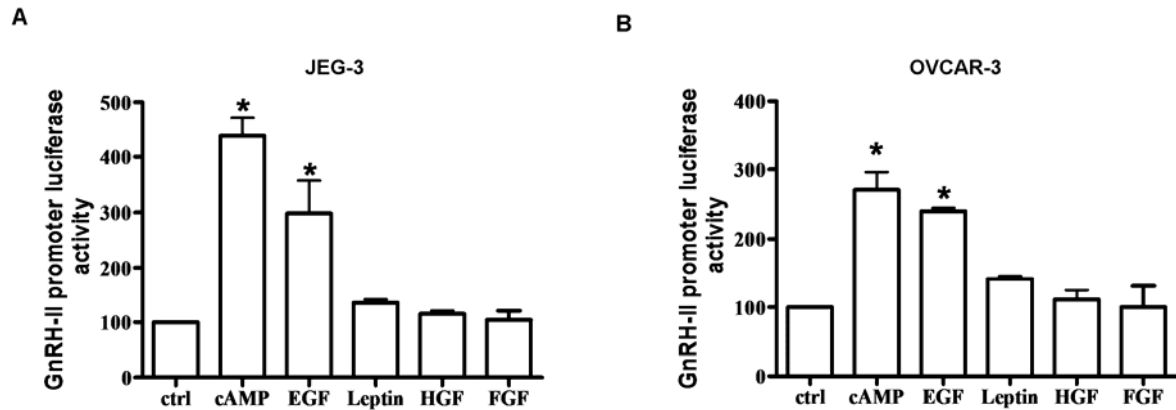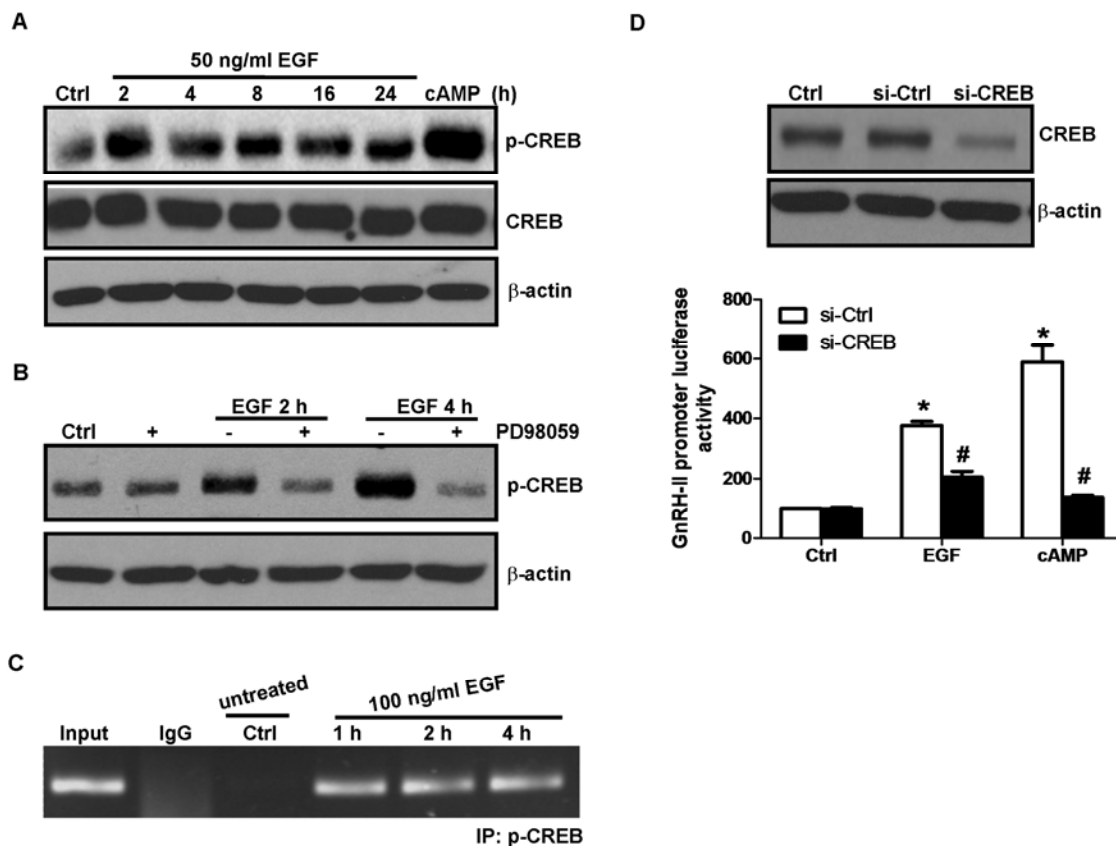

(A) JEG-3 cells and (B) OVCAR-3 cells were treated with 8-bromo cAMP, EGF, leptin, HGF and FGF for 24 h after transient transfection with a *GnRH-II* promoter luciferase construct together with a (RSV)-*lacZ* plasmid. Cell lysates were collected for luciferase assay and measurements of β-galactosidase activity as a control for transfection efficiency. Results are expressed as mean ± SEM luciferase activity/β-galactosidase activity (i.e. GnRH-II promoter luciferase activity) of three independent experiments. * $p<0.05$ compared to untreated control (ctrl).

Appendix II. EGF induces *GnRH-II* promoter activity and GnRH-II mRNA levels in JEG-3 choriocarcinoma cells.
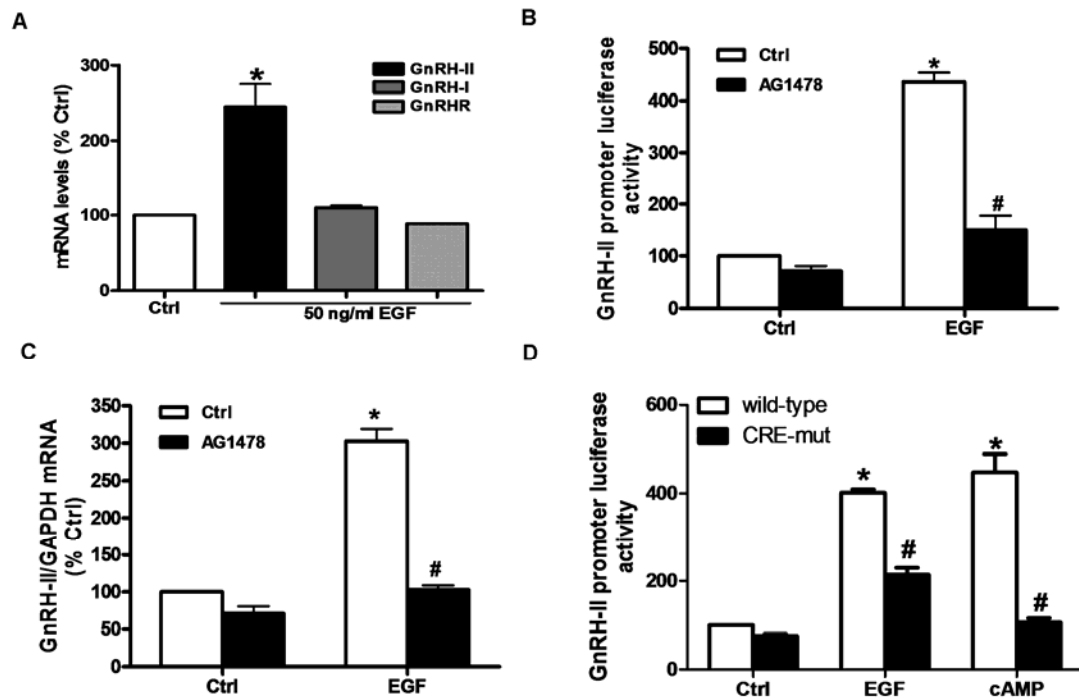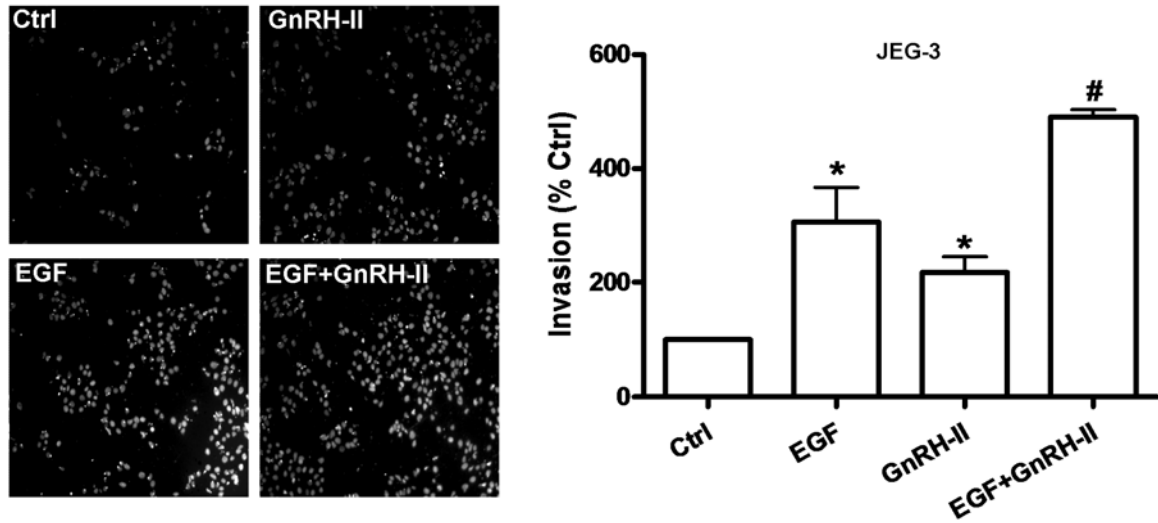

(A) JEG-3 cells were treated with 50 ng/ml of EGF for 24 h and total RNA was used to prepare cDNA for RTq-PCR to evaluate the effect of EGF on GnRH-II, GnRH-I and GnRHR mRNA levels expressed as percentage over untreated control (Ctrl). Results are expressed as mean± SEM of three independent experiments. * $p<0.05$ compared to untreated control (Ctrl). (B) JEG-3 cells were transiently transfected with a *GnRH-II* promoter-driven luciferase reporter gene construct together with a (RSV)-*lacZ* plasmid and then pre-treated with 10 μM EGFR inhibitor (AG1478) for 30 min prior treatment with 50 ng/ml EGF for 24 h. Cell lysates were collected for luciferase assay and measurements of β-galactosidase activity as a control for transfection efficiency. Results are expressed as mean ± SEM luciferase activity/β-galactosidase activity (i.e. GnRH-II promoter luciferase activity) of three independent experiments.* $p<0.05$ compared to untreated control (Ctrl). # $p<0.05$ compared to cells treated with EGF. (C) In parallel experiments, total RNA was used to prepare cDNA after administration of AG1478 in the presence or absence of 50 ng/ml EGF for 24 h and subjected for RTq-PCR to evaluate the effect of EGF on GnRH-II mRNA levels expressed as percentage over untreated control (Ctrl). Results are expressed as mean± SEM of three independent experiments. * $p<0.05$ compared to untreated control (Ctrl). # $p<0.05$ compared to cells treated with EGF. (D) JEG-3 cells were transfected with wild type *GnRH-II* promoter-driven lucifease reporter gene construct or a 3 bp mutated CRE-*GnRH-II* promoter-driven luciferase reporter construct followed by 50 ng/ml EGF or 1 mM cAMP treatment. Cell lysates were collected for luciferase assay and measurements of β-galactosidase activity as a control for transfection efficiency. Results are expressed as mean ± SEM luciferase activity/β-galactosidase activity (i.e., GnRH-II promoter luciferase activity) of three independent experiments.* $p<0.05$ compared to untreated control (Ctrl). # $p<0.05$ compared to cells transfected with wild type *GnRH-II* promoter-driven luciferase reporter gene contruct and followed by EGF or cAMP treatment.

Appendix III. EGF induced phosphorylation of CREB activates cAMP-responsive element within the human *GnRH-II* promoter.


(A) Nuclear lysates harvested from EGF treated cells were subjected to Western blotting and probed for phosphorylated CREB (p-CREB). Total CREB (CREB) and β-actin were used as normalization control. (B) JEG-3 cells were pre-treated with 20 μM PD98059 (selective MAPK inhibitor) for 30 min and then co-treated in the presence or absence of 50 ng/ml EGF for 2 h or 4 h. Nuclear cell lysates were collected and the level of p-CREB was determined by Western blotting. β-actin was used as normalization control. (C) JEG-3 cells were treated with 50 ng/ml EGF for 1 h, 2 h, 4 h, or were untreated (Ctrl). Cross-linked, sheared chromatin was immuno-precipitated (IP) with p-CREB and recovered chromatin was subjected to PCR analysis using primers spanning the CRE region of the *GnRH-II* promoter. The IgG lanes are ChIPs performed using non-specific IgG. An ethidium bromide-stained gel of PCR products shows a representative of ChIP analysis from three independent experiments. (D) The efficiency of the siRNA for CREB was tested by Western blotting (Upper panel). Cells were transfected with *GnRH-II* promoter-driven luciferase reporter gene construct together with 150 nM random control siRNA (si-Ctrl) or CREB siRNA (si-CREB) for 24 h, and then treated with 50 ng/ml EGF for 24 h. Cell lysates were assayed for luciferase activity and measurements of β-galactosidase activity as a control for transfection efficiency, the result of which are expressed as mean ± SEM luciferase activity/β-galactosidase activity (i.e. GnRH-II promoter luciferase activity) of three independent experiments.* $p<0.05$ compared to cells treated with a control siRNA (si-Ctrl). # $p<0.05$ compared to cells treated with CREB siRNA and followed by EGF or cAMP treatment.

Appendix IV. EGF and GnRH-II additively enhances JEG-3 cell invasive potential


JEG-3 cells were treated with 25 ng/ml of EGF, 10 nM of GnRH-II or in combination for 24 h and then seeded into Matrigel-coated transwells and cultured for 48 h. Results are expressed as mean ± SEM of three independent experiments. *$p<0.05$ compared with untreated control (Ctrl). # $p<0.05$ compared with EGF or GnRH-II treatment.

Appendix V. LRP regulates N-cadherin expression to enhance ovarian cancer cell invasiveness.
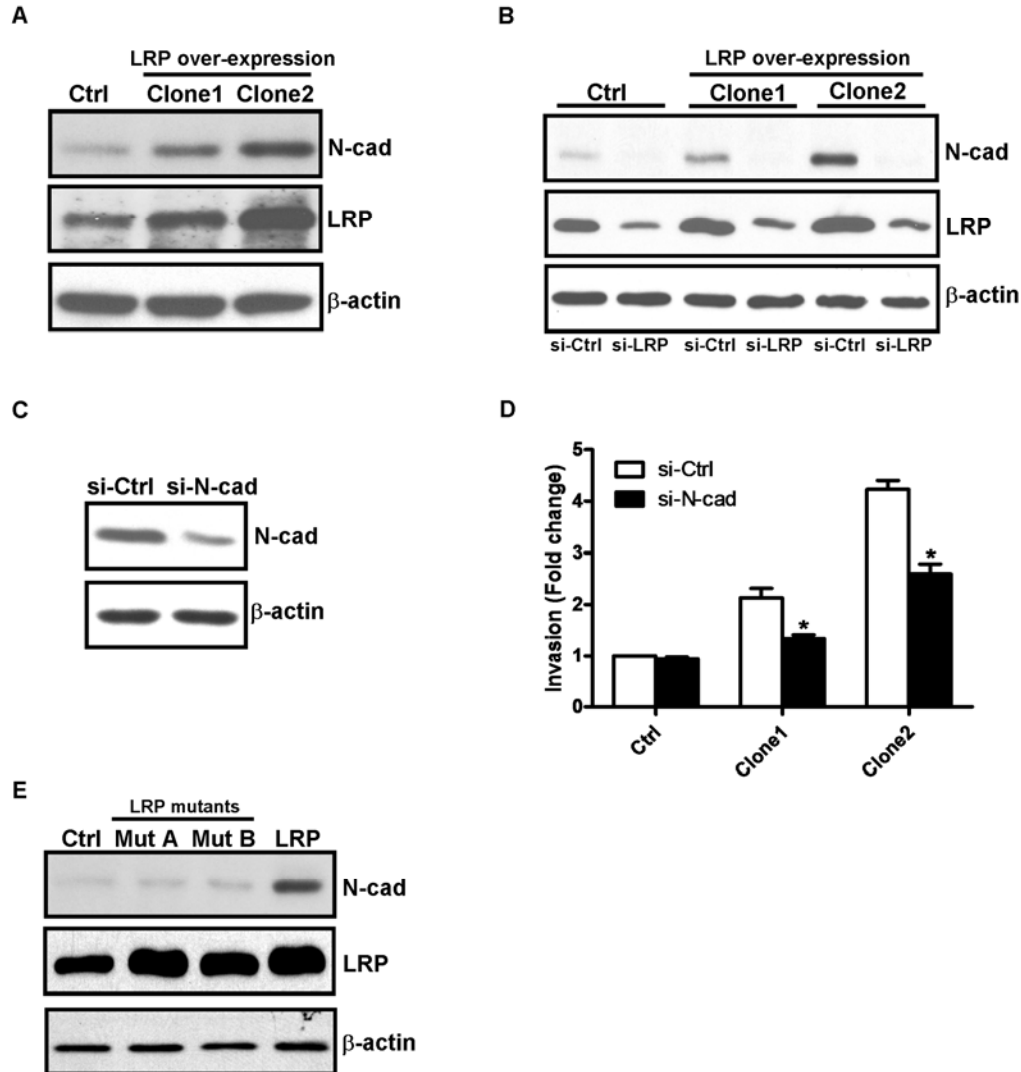
CaOV-3 cells were stably transfected with expression vectors for wild type LRP, two different LRP mutants, or eGFP as a control (Ctrl). (A) The efficiency of LRP over-expression and the N-cadherin levels (N-cad) in independent clones (i.e. Clones 1 and 2) were tested by Western blotting. β-actin was probed as a normalization control. (B) In parallel experiments, these stably transfected CaOV-3 cells were treated with 75 nM LRP siRNA (si-LRP) or 75 nM control siRNA (si-Ctrl). Protein lysates were then harvested and subjected to Western blotting to monitor N-cad protein levels. (C) The efficiency of 50 nM N-cad siRNA (si-N-cad) was tested in Western blotting. (D) LRP over-expressing CaOV-3 cells and control cells (Ctrl) were treated with 50 nM si-N-cad or 50 nM si-Ctrl prior subjected to invasion assay for 48 h. Results are expressed as mean ± SEM of three independent experiments. *$p<0.05$ compared with siRNA control (si-Ctrl). (E) Protein lysates of CaOV-3 cells stably over-expressing wild type LRP, two different LRP mutants (i.e. Mut A and Mut B) and eGFP control vector (Ctrl) were harvested and subjected to Western blotting for their relative N-cad protein levels or β-actin as a normalization control.

Appendix VI. GnRH-II-induced MMP-2 and MT1-MMP in two independent signaling pathways.
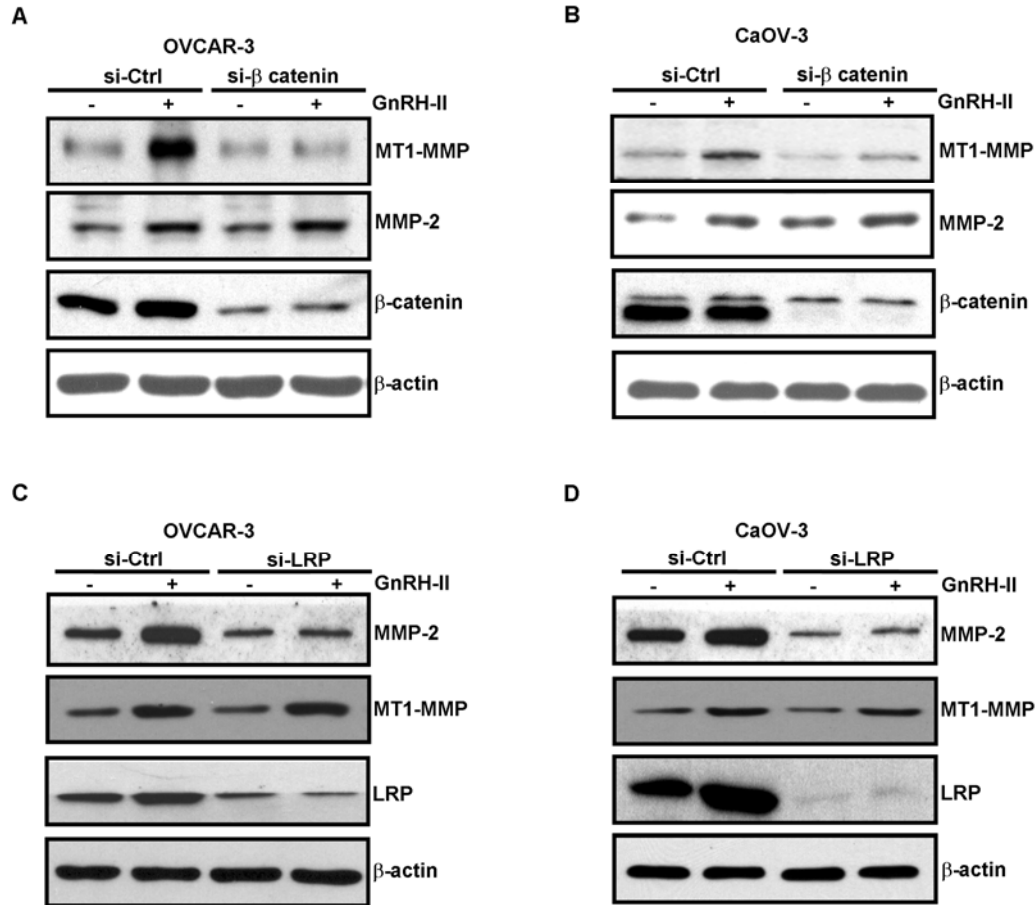
(A) OVCAR-3 and (B) CaOV-3 cells were treated with 50 nM β-catenin siRNA (si-β-catenin) or 50 nM control siRNA (si-Ctrl) for 24 h and then treated with 10 nM GnRH-II for 24 h. Cell lysates were harvested and subjected to Western blotting with antibodies against MMP-2, MT1-MMP, β-catenin, or β-actin as the normalization control. (C) OVCAR-3 and (D) CaOV-3 cells were treated with 75 nM LRP siRNA (si-LRP) or 75 nM siRNA control (si-Ctrl) for 24 h prior treatment with 10 nM GnRH-II for 24 h. Cell lysates were then harvested and subjected to Western blotting with antibodies against MMP-2, MT1-MMP, LRP, or β-actin as the normalization control. Western blot results were representative blots of three independent experiments.