THE EFFECTS OF GONADOTROPIN RELEASING HORMONE-I AND II IN HUMAN GRANULOSA CELLS

by

In-Sun Hong

B.Sc., Kyonggi University, 2002

M.Sc., Seoul National University, 2004

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Abstract

Gonadotropin-releasing hormone (GnRH) is the key regulator of hypothalamus-pituitary-gonadal function. In the anterior pituitary, GnRH stimulates the synthesis and the release of the gonadotropins. GnRH and its receptors have also been found in extra-pituitary tissues such as the ovary, breast, and placenta. GnRH has been reported to have anti-proliferative effects on granulosa cells from rats, pigs, and humans; however, the underlying mechanisms are unclear.

We have successfully established two immortalized human granulosa cell lines by SV40 large T antigen transfection. In the process, we have characterized multiple cellular and molecular features of these immortalized cells and compared these characteristics with those of primary cultured granulosa cells. The features examined include the expression patterns of cytoskeletal proteins (vimentin, cytokeratin 5/6 and desmin), adhesion molecules (Connexin 43 and E-cadherin), steroidogenic enzymes (StAR, p450scc and 3β-HSD), steroid receptors (ER-α, -β and PR-α, -β) and gonadotropin receptors (LH and FSH), as well as cell morphology, anchorage-independent growth, growth potential, and progesterone production.

In addition to its well-established actions on the pituitary–gonadal axis, GnRH-I

or II suppressed proliferation and directly induced apoptosis in human granulosa cells. The intracellular signals of apoptosis induced by GnRH-I or II, in turn, are mediated by activation of the proteolyic caspase cascade, involving caspase-8, -3 and -7. In addition, FSH protected human granulosa cells from undergoing apoptosis induced by GnRH-I or II.

GnRH-I or II also significantly attenuated the stimulatory effect of IGF-I on granulosa cell proliferation. Correspondingly, IGF-I decreased while GnRH-I or II increased cleaved caspases-3 protein expression in our granulosa cell line; furthermore, IGF-I significantly attenuated the expression of cleaved caspase 3 induced by GnRH-I or II. These interactions of IGF-I and GnRH-I or II in regulating granulosa cell proliferation or apoptosis are mediated through Akt in human granulosa cells.

In conclusion, we established two granulosa cell lines and performed integrated investigations of multiple characteristics to elucidate more clearly the characteristics of human ovarian granulosa cells. The present study demonstrated that GnRH-I or II mediates anti-proliferate effects via stimulation of granulosa cell apoptosis and inhibition of IGF-I activities through Akt signaling pathway in human granulosa cells.

Preface

A version of chapter 4 has been published. [In Sun, Hong], Hong IS, Cheung AP, Leung PC 2008 Gonadotropin-releasing hormones I and II induce apoptosis in human granulosa cells. J Clin Endocrinol Metab 93:3179-3185. I conducted all the experiment and wrote most of the manuscript. As a co-author, Dr. Anthony Cheung has provided technical supports such as statistics and revised this manuscript. As a supervisor, Dr. Peter C.K. Leung has provided overall ideas and discussions in this manuscript.

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AIG, Anchorage-Independent Growth

APAF-1, Apoptosis activation Factor 1

ART, Assisted Reproduction Technology

BCL-2, B cell/lymphoma-2

BSA, Bovine Serum Albumin

CPDL, Cumulative Population Doubling Levels

CREB, CRE-Binding protein

CYP11A1, gene encodes the cholesterol side-chain cleavage enzyme (P450scc)

CYP17, gene encodes the Hydroxylase/17,20 Lyase.

CYP19, gene encodes the aromatase

CX43, Connexin 43

BMP, Bone Morphogenetic Protein

DAG, Diacylglycerol

DD, Death Domain

ECL, Extracellular Loop

EGF, Epidermal Growth Factor

ER, Estrogen Receptor

FADD, Fas-associated death domain

FGF, Fibroblast Growth Factor

FSH, Follicle Stimulating Hormone

GDF, Growth Differentiation Factor

GnRH, Gonadotropin-Releasing Hormone

GPCR, G Protein-Coupled Receptor

HCG, Human Chorionic Gonadotrophin

HGF, Hepatocyte Growth Fator

HPV, Human Papillomavirus

IGF-I, Insulin-like growth factor-I

IGFBP, Insulin-like growth factor Binding Protein

IHG, Immortalized Human Granulosa cells

IP_3, Inositol 1,4,5-trisphosphate

IVF, In Vitro Fertilisation

JNK, c-Jun N-terminal Kinase

KGF, Keratinocyte Growth Factor

KL, Kit ligand

LH, Luteinizing Hormone

MAPK, Mitogen-activated protein kinases

MMP, Matrix Metalloproteinases

NFκB, Nuclear Factor Kappa B

OSE, Ovarian Surface E pithelial

PAI, Plasminogen Activator Inhibitor

PARP, Poly (ADP-ribose) Polymerase

PBS, Phosphate-Buffered Solution

PKC, Protein Kinase C

PLC, Phospholipase C

RIP, Receptor Interacting Protein

PR, Progesterone Receptor

RT-PCR, Real Time-Polymerase Chain Reaction

SL/DT Scrape Loading and Dye Transfer

SAPK, Stress Activated Protein Kinase

StAR, Steroidogenic Acute Regulatory Protein

SVOG, SV40 transformed ovarian granulosa cell lines

TGF, Transforming Growth Factor

TNF, Tumor Necrosis Factor

TRADD, TNF receptor-associated death domain

TRAF, TNF-receptor associated factor

TRAIL, Tumor necrosis (TNF)-Related Apoptosis-Inducing Ligand

TUNEL, terminal deoxynucleotidyl-transferase (TdT)-mediated biotin-dUTP nick-end labeling

3ß-HSD, ***3***-β-Hydroxysteroid Dehydrogenase

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1. Literature review

1.1. Follicular development

1.1.1. Primordial follicles

After 4 weeks of pregnancy, the mesodermal precursors of ovarian follicles migrate from the wall of the yolk sac to the primitive gonadal fold and proliferate to a total number of ~7 x 10^6 cells (1). In the latter half of pregnancy, the total oocyte number decreases to 1 x 10^6 (1). Total oocyte number is rapidly decreased before birth by apoptosis. Apoptosis of the oocyte occurs most frequently between 14 and 28 weeks, but the apoptotic rate decreases gradually after that period (1). After birth, apoptosis continues to occur among oocytes until adolescence, eliminating 75% of the initial ~7 x 10^6 cells (1). During early embryonic development, oocyte proliferation is not associated with that of somatic cells (2). The formation of primordial follicles occurs during the later period of embryonic development. Primordial follicles comprise one oocyte, incompletely surrounded by a granulosa cell layer without theca cell layers (3). In general, a woman is born with about two million primordial follicles in her ovaries. Most primordial follicles in the ovary remain in a quiescent state with oocytes exhibiting arrested meiosis until adolescence. In sexually mature females, the transition from primordial to primary follicle can be initiated and meiotic division is initiated in

each oocyte (4-7).

1.1.2. Primordial to primary follicle transition

One of the important changes in the early development of primordial follicle is a morphological change in granulosa cell layers from a flat to a cuboidal structure (8). The primary follicles developing from primordial follicles exhibit increased oocyte diameter and are surrounded by a complete single-granulosa cell layer that does not include theca cells (3, 7-9). Another prominent early event in the primary follicular development process is the formation of the first theca cell layer from the mesenchymal stroma and increased proliferation of both theca and granulosa layers in individual follicles (3, 7-9). At this phase of follicular growth, gonadotropins (LH or FSH) do not appear to be involved in the proliferation of either granulosa or theca cells. The granulosa and theca cells in the primary follicle do not respond to gonadotropins (LH and FSH) (7). Previous studies have suggested that several growth factors are involved in precursor cell recruitment and proliferation at this stage of follicular development (10, 11). According to previous studies, theca cells produce many growth factors such as transforming growth factor (TGF)-α, hepatocyte growth factor (HGF) and keratinocyte growth factor (KGF), that can regulate the proliferation of granulosa

cells (10, 11), while granulosa cells produce Kit ligand (KL), which can be involved in theca cell proliferation and oocyte differ entiation (11).

1.1.3. Folliclar growth and development throughout adult life

During a woman's reproductive years, a number of primordial follicles begin to grow during each menstrual cycle. From a 300,000-large reservoir, about ~450 follicles are ovulated during the reproductive years (12-14). The development of a primordial follicle to a preovulatory follicle takes around 120 days (15).

1.1.3.1. Primary follicle

The early primary follicle consists of single oocyte surrounded by a flattened layer of granulosa cells (16). Notably, the gap junction plays an important role in the connection between the oocyte and surrounding granulosa cells by transporting growth factors and lipid precursors into the oocyte (17). At this stage in development, the zona pellucida begins to form between granulosa cells and the oocyte (18). The zona pellucida, a thick layer of glycoprotein and proteoglycans, is mainly produced and released by granulosa cells (19). The regulatory mechanism for the development of primary follicles is not yet clearly established. Oocyte-granulosa cell layer

communication is essential for the development of primary follicles (17). The growth and differentiation of primary follicles probably depends on oocyte-granulosa cell layer communication and the secretion of many locally produced factors by granulosa cells (20-22). For example, BMP (bone morphogenetic protein) -15 has been shown to be essential for the growth of primary follicles (23). Mutations in the BMP-15 gene induce follicular growth arrest and cause infertility in female sheep (23). GDF-9 is produced by the oocyte itself, and implicated in oocyte growth in primary follicles. GDF-9 has been localized in the oocyte; moreover, active immunization against GDF-9 arrests follicular development at the primary follicle stage in female sheep (24).

1.1.3.2. Antral follicle growth

Once an oocyte has stopped growing, granulosa cells begin to proliferate and form a multiple-granulosa cell layer (25). This multiple granulosa cell layer is properly separated from the theca cell layer by a basement membrane known as the basal lamina (25). Antral follicle growth depends on gonadotropin (LH and FSH) concentrations, and antral follicle growth is associated with increased estrogen production (26). In addition, the specific receptor for FSH is not detected in granulosa cells until the antral stage. FSH is known to stimulate steroidogenesis (estrogen

production) in granulosa cells and initiates the proliferation of granulosa cells (26). The stimulatory action of FSH on aromatase is required for the conversion of androgens to estrogens and is an essential factor for the regulation of estrogen production in the ovary. The stimulating effect of FSH on steroidogenic enzyme activities including those of 3ß-HSD, P450scc and P450arom in ovarian granulosa cells was investigated in many species (27, 28). A number of locally produced factors are also involved in follicle development. It has been suggested that BMP can regulate steroidogenesis and *in vitro* proliferation in bovine granulosa cells (29). IGF-I is necessary for follicular differentiation because it enhances the effects of gonadotropins (30). The expression of IGF-I receptor and IGFBPs (IGFBP-2, -3, and -4) at this developmental stage has been reported (31).

1.1.4. Follicle selection and dominance

The precise mechanisms of dominant follicle selection and the maintenance of dominance need to be fully elucidated. It has been suggested that a decline in FSH concentration may be a key regulator in dominant follicle selection (26). Between the first and fourth days of the menstrual cycle, a number of follicles are recruited from a resting pool of primordial follicles in response to FSH (26). All recruited follicles seem

to be involved in the early decrease in peripheral FSH concentrations (32). The largest follicle regulates a further decrease in FSH concentration; below this threshold, other smaller follicles cannot continue to grow (28, 33, 34). Only one follicle is selected from a number of recruited follicles, and this selected follicle becomes the dominant follicle that will ovulate between the 12th and 14th days from first day of memses (35). The remaining subordinate follicles stop growing and begin to undergo follicular atresia. The expression of LH receptor and 3ß-HSD mRNAs was detected in granulosa cells at about the time of dominant follicle selection (27, 28), supporting the idea that LH may be involved in continuous growth of the dominant follicle when peripheral FSH levels are decreasing. Sufficient circulating levels of LH are essential for the extended lifespan of the dominant follicle during the late follicular phase (36). These regulatory effects of gonadotripins (LH or FSH) on follicular development and selection of the dominant follicle are probably mediated by locally synthesized growth factors such as TGFß, FGF, and IGF-I (37). For instance, it has been suggested that the insulin/IGF-I system regulates FSH-mediated estrogen production in bovine granulosa cells (38, 39). However, the precise roles of locally produced growth factors in selection and dominance remain to be elucidated.

1.1.5. Follicular atresia

During the early phase of the menstrual cycle when FSH level is increased with the development of cell, dominant follicle synthesizes large amounts of estrogen, which in turn decrease FSH production through a negative feedback mechanism (40). Therefore, the smaller subordinate follicles gradually undergo apoptosis; this hormonally controlled apoptotic process is known as follicular atresia (12, 41). Certainly, the first prominent event in the process of follicular atresia is a decrease in FSH receptor expression in the granulosa cell layers (42). Indeed, the decrease in FSH concentration is important for follicular atresia, but steroid hormones and growth factors are also involved in this apoptotic process (42). Tumor necrosis factor (TNF), locally synthesized in the follicular granulosa cells, suppresses FSH-mediated estrogen production within a follicle, except in the dominant follicle (43). Furthermore, TNF expression was inversely correlated with FSH concentration and FSH-mediated steroid hormone production in granulosa cells (43).

1.1.6. Luteolysis

Three days after ovulation, granulosa cells begin to increase in size. These enlarged granulosa cells associate with the newly differentiated theca-lutein cells from

surrounding theca cell layers, and the surrounding stroma cell layer becomes part of the corpus luteum (44). In addition to luteal cells, the corpus luteum also includes endothelial cells, leukocytes, and fibroblasts (45). These non-steroidogenic cells occupy 70-80% of the entire cell population in the corpus luteum. The luteal cell population is mainly composed of two sub-types of luteal cells (larger and smaller) with different morphology and function (45). Larger luteal cells, presumably derived from granulosa cells, show greater steroidogenic potential than smaller luteal cells (46). Approximately 10 days after ovulation, the corpus luteum rapidly begins to decline; this phenomenon is known as luteolysis (47). However, the precise mechanisms of corpus luteum degradation in the primate menstrual cycle remain unknown. Luteolysis is characterized by a rapid decline of progesterone production that is usually designated as the functional process of luteolysis (48). Alternatively, morphological degradation of the corpus luteum is accompanied by overall structural changes (47). A number of factors have been suggested as regulators of the process of luteolysis. Structural regression of the corpus luteum is correlated with increased estrogen and decreased progesterone levels during the late luteal phase (49). The luteal cell possesses gap junctions, which are essential for communication and the exchange of apoptotic signals between luteal cells (50). Marked structural changes in the corpus

luteum during luteolysis are associated with enhanced matrix metalloproteinase (MMP) expression and activity (51).

1.2. Gonadotropin-releasing hormone (GnRH)

1.2.1. Structure

Gonadotropin-releasing hormone (GnRH) is isolated as a decapeptide (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) from the hypothalamus and plays an important regulatory role in the reproductive hormonal system (52, 53). It is synthesized in the median eminence region of the hypothalamus and released into the anterior pituitary gland through the portal vascular system. The human GnRH-I gene has been located on the short arm of chromosome 8. The gene encoding GnRH-I is about 4300 bp in length, with four exons separated by three large introns: exon I corresponding to the 5' untranslated region; exon II corresponding to a 23-residue N-terminal signal peptide; 10 amino acids for GnRH decapeptide, which includes a 3-amino-acid signal sequence and the first 11 amino acids of the GnRH-associated peptide (GAP); exon III corresponding to amino acids 12 to 43 of GAP; and exon IV corresponding to the remainder of GAP (54). GnRH-I is synthesized as a 92-amino-acid pro-peptide, which is followed by: 1) a 23-residue N-terminal signal peptide; 2)

the 10 amino-acid sequence for GnRH decapeptide itself; 3) a 3-amino-acid sequence essential for the processing signal; and 4) a 56-residue C-terminal peptide, which is denoted as GnRH-associated peptide (GAP) (55). The N-terminal four amino acids and C-terminal three amino acids have been conserved over at least 600 million years of evolution (56). GnRH exhibit considerable variation in positions 5-8, and these residues are important for receptor-binding affinity. Therefore, GnRH is a key factor in determining receptor selectivity (57, 58). The second form of GnRH was first isolated from chicken brains (58-60). In humans, the gene encoding GnRH-II is located on the chromosome 20p13 (61). It also comprises four exons separated by three introns, and the precursor proteins for human GnRH-II are mostly similar to those for type I GnRH. Interestingly, human GnRH-II mRNA is expressed most strongly outside of the brain.

1.2.2. GnRH secretion

Because the half-life of endogenous GnRH in the peripheral circulation is only 2-4 min (62), a biologically meaningful amount of GnRH cannot escape from the hypophyseal portal system. Therefore, the regulation of stable GnRH secretion is important for normal reproductive function. The release of GnRH is controlled by complicated interactions of various elements such as neurohormones, gonadotropins

and steroid hormones. These interactions are regulated by negative and positive feedback (63). The central nervous system regulates GnRH secretion through finely tuned control of various neurotransmitters such as dopamine, norepinephrine, endorphin, serotonin, and melatonin (64, 65). Since the estrogen receptor is not expressed in GnRH-releasing neurons (66), steroid hormonal regulation for GnRH secretion appears to be an indirect effect, mediated by the regulation of various neurotransmitters. Because gonadotropins (LH and FSH) are secreted in a pulsatile pattern, the pulsatile pattern of GnRH secretion from hypothalamus can be predicted by measuring the LH secretion pattern. The long half-life of FSH in the peripheral circulation has made it difficult to use FSH as a reliable indicator of GnRH secretion (67). The pulsatile patterns of LH secretion have been measured during the follicular phase and luteal phase (68). During the follicular phase, the frequency of LH secretion was relatively high, whereas the amplitude of LH concentration was low in comparison with that observed during the luteal phase. It has been suggested that the pulsatile pattern of GnRH secretion is related to the differential synthesis of LH and FSH (69). High-frequency GnRH pulses (10 min) selectively stimulate the production of common α and LHβ mRNAs during ovulation, while low frequencies (120 min) increase FSHβ mRNA production during the follicular phase.

1.2.3. Distribution

1.2.3.1. Brain

The most important difference between GnRH-I and GnRH-II expression patterns in multiple human tissues is that GnRH-I is not highly expressed outside the brain regions (61). In humans, GnRH-I neuron cell bodies are concentrically distributed in the preoptic area and basal hypothalamic area (70). Interestingly, the expression of mRNA encoding human GnRH-II in the kidney is 30 times stronger than in any region of the brain and its expression in normal human bone marrow and prostate is approximately four-fold higher than in the brain (61).

1.2.3.2. Ovary

The expression of both GnRH-I and II mRNA in various human ovarian cell types including granulosa cells from *in vitro* fertilization (IVF) patients (71) and ovarian surface epithelial (OSE) cells (72) has been demonstrated in previous studies. The expression of GnRH receptor mRNA has been investigated in the ovary of many species, including human (73-75). The primary sequence of ovarian GnRH receptor has previously been reported; its sequence is identical to those found in the pituitary

GnRH receptor in rat (76) and human (77) granulosa cells. In the ovary, GnRH may play an autocrine and/or paracrine regulatory role by modulating the effects of gonadotropins and growth factors. It has been shown that GnRH induces anti-proliferative effects in pig granulosa cells (78) and pro-apoptotic effects in rat granulosa cells (79). Several studies suggest a physiologic role of GnRH in the regulation of follicular atresia and luteolysis (80). GnRH can regulate hormonal biosynthesis in rat and human granulosa cells (81, 82), and many studies reported the inhibitory effect of GnRH on steroidogenesis in human granulosa cells (83). GnRH analogue administration has been shown to reduce the expression of luteinizing hormone (LH) receptor (84), follicle-stimulating hormone (FSH) receptor (81), and aromatase enzyme (85) in rat granulosa cells.

1.2.3.3. Placenta

The presence of GnRH in the human placenta has been recognized in previous studies (86). GnRH has been found to stimulate human chorionic gonadotrophin (HCG) production in the human placenta, in a dose-dependent manner (87). The presence of GnRH receptor mRNA and protein in cultured primary human trophoblast cells has been reported (88). These results suggest the existence of GnRH-mediated

regulatory systems for gonadotrophin production in human placenta. According to recent results from several studies, GnRH-I and GnRH-II improved the invasive capacity of human trophoblasts by regulating key matrix metalloproteinases (MMPs), such as MMP-2 and MMP-9, as well as uPA/PAI protease systems (89, 90).

1.2.3.4. Breast

Previous studies have shown that the mRNAs of two forms of GnRH are widely expressed in normal human breast tissue and also over-expressed in human breast tumor tissue (91). GnRH receptor mRNA has also been found in various human breast tumor cell lines (MCF-7 and MDA-MB 468) (92). GnRH can cause regression of hormone-dependent breast tumors, due to their inhibition of steroid hormone production (93). The exact action mechanism of GnRH in normal and tumor breast has not yet been established, but many studies have shown that GnRH-I and II mediated direct anti-proliferative effects in breast tumors (94). Therefore, we can postulate that GnRH may exert anti-proliferative effects in breast tumors through both direct and indirect pathways. The molecular mechanisms for the direct antiproliferative effects of GnRH agonists on breast tumors are thought to attenuate the effect of growth factor (IGF-I and EGF)-mediated mitogenic signaling pathways (95, 96).

1.2.3.5. Uterus

The expression of GnRH-I mRNA has been established in almost every cell type in human uterus (97-99). Interestingly, the expression pattern of human GnRH mRNA varies remarkably; depending on the menstrual cycle. The highest levels are observed during the secretory phase as compared to the proliferative phase (100, 101). The expression of GnRH-I protein has also been detected in all endometrial cell types; the highest level of expression was observed during the luteal phase (101). GnRH-II also exhibits a dynamic expression pattern throughout the human menstrual cycle: a higher level of immunoreactivity was detected during the secretory phases as compared to the proliferative phases (102). This suggests that increased expression of GnRH-I and/or GnRH-II protein during the early and mid-secretory phase may play a regulatory role in embryo implantation (102).

1.2.4. Characteristics of GnRH receptor

The three dimensional structure of the GnRH-I receptor is important for an understanding of its ultimate cellular and physiological function. The sequence of the mannalian GnRH-I receptor was initially established in the mouse pituitary cell line,

αT3 (103). Additionally, GnRH-I receptor sequence was identified with 80% amino acid similarity in other mammals such as rat (104), pig (105), cow (106), sheep (107) and human (108). Gene cloning technology has revealed the presence of highly selective type II GnRH receptor in amphibian (109) and primate (110). The amino acid sequence of GnRH-II receptor has 68% similarity to the GnRH-I receptor in the marmoset (110). The most obvious difference between the GnRH-I and -II receptors is the presence of a 56-amino-acid cytoplasmic tail at the C-terminal of the GnRH-II receptor, as compared with its absence in GnRH-I receptor (111). The cytoplasmic tail domain of the GnRH-II receptor is involved in receptor trafficking. According to a recent study, GnRH-II receptor from the marmoset undergoes more rapid GnRH analogue-mediated receptor internalization than does the human GnRH-I receptor (112). Other significant structural differences between GnRH-I and II receptors are found in the cytoplasmic, transmembrane and extracellular loop domains. The extracellular domains and surface regions of the transmembrane domains are typically involved in the ligand-binding activity of both GnRH-I and -II receptors (113). The N-terminal domain of GnRH-II receptors is more negatively charged and two amino acids longer than that in the GnRH I receptor (114). Extracellular loop 1 (ECL-1) displays an almost similar electrical charge in both mammalian GnRH-I and -II

receptors, but ECL-2 is relatively more positively charged in GnRH-II receptors as compared to GnRH-I receptors. Furthermore, ECL-2 and ECL-3 of GnRH-II receptor are shorter than those in GnRH-I receptor (114). These differences seem to be involved in receptor structure and conformational changes associated with subsequent receptor-mediated signal transduction. However, the functional full-length GnRH-II receptor is not present in horse, cow, rat, sheep, mouse, chimpanzee, or human, in spite of its expression in pig, marmoset and rhesus monkey (115).

1.2.5. Signaling transduction of GnRH receptor

1.2.5.1. G protein coupling

The receptor for GnRHs belongs to the G protein-coupled receptor (GPCR) superfamily (116). GPCR, one of the largest group of trans-membrane receptors, is structurally known as 7-transmembrane spanning helices and connected by consecutive intracellular and extracellular loops, which may be glycosylated (117). The extracellular and transmembrane domain regions are involved in ligand-binding activity of both GnRH-I and -II receptors (113), whereas the cytoplasmic regions provide the interaction sites for G-proteins and other intracellular regulatory proteins (113). G-proteins consist of Gα, Gβ, and Gγ subunits. Agonist binding to the GPCR

induces activation of Gα proteins, resulting in GDP/GTP exchange on the Gα subunit. Gα-GTP subunit is released from Gβγ and stimulates various downstream signaling molecules. It has been reported that GnRH-I receptor interacts directly with the $G\alpha_{q/11}$ subunit in pituitary gonadotropes (116, 118). GnRH exerts multiple functional effects in pituitary gonadotropes, such as the production and secretion of gonadotropins (LH and FSH) (119). In gonadotropes, GnRH is able to stimulate phospholipase Cβ (PLCβ), resulting in the cleavage of phosphoinositides (PIP_2) to generate the intracellular signaling molecules inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG), which lead to intracellular calcium (Ca^{2+}) signaling and activation of the protein kinase C (PKC) signaling pathway, respectively (120).

1.2.5.2. Protein kinase c

Protein kinase c (PKC) is a family of serine/threonine lipid-activated kinases (121, 122). PKC is a family consisting of at least 10 structurally related isoforms (123). PKC might be involved in the regulation of cellular growth, differentiation, exocytosis, apoptosis and cell cycle control (124). The isoforms of PKC are classified into the following three groups: conventional PKC (cPKC-α, -βI, -βII, and -γ), novel PKC (nPKC-δ, -ε, -θ, and -*η*/L) and atypical PKC (aPKC-*ζ* and -*ι*/*λ*) (124). The structure of

each PKC consists of a regulatory domain, a catalytic domain and a hinge region. The flexible hinge region connects the regulatory domain and the catalytic domain (124). GnRH is able to increase mRNA levels of PKCβ, PKCδ and PKCε (but not PKCα) in a Ca^{2+}-dependent manner in αT3-1 cells (125, 126). Additionally, GnRH agonist-mediated expression levels of PKCβ, PKCδ and PKCε seem to be regulated by the PKC signaling pathway itself, indicating auto-regulation of PKC gene expression (125, 127).

1.2.5.3. Mitogen-activated protein kinases (MAPK)

MAPK signaling cascades regulate differentiation, proliferation, migration, and apoptosis in mammals (128). Three main MAPK subfamilies are evolutionarily conserved from yeast to mammals: ERK1/2 (p42 and p44), JNK 1–3 and p38α-δ (128). The ERK1/2 signaling pathway is stimulated in response to various growth factors and mitogens (128). The phosphorylation of ERK1/2 by upstream activators MEK1 and MEK2 induces a conformational change and detachment of ERK1/2 from MEK1/2, enabling translocation of activated ERK1/2 from the cytoplasm to the nucleus. After entry into the cell nucleus, phosphorylated ERK1/2 can activate multiple transcription factors such as Elk-1, c-Jun, c-Fos, and CREB (129). JNK1/2/3 (c-Jun N-terminal

kinase) is also known as stress-activated protein kinase (SAPK1). The predominant isoforms are JNK1 (46-KD) and JNK2 (55-KD), which exhibits 83% identity to JNK1 (130). Despite their high degree of similarity, the two JNK isoforms differ greatly in terms of their ability to bind c-Jun. JNK2 binds c-Jun 25 fold more efficiently than does JNK1 (130). JNK can be activated by a number of cellular signals including growth factors, cytokines, and extracellular stress (128, 129). JNK activates a variety of target transcription factors such as c-Jun, ATF-2, HSF-1, STAT3 and others that mediate extracellular stress responses, inflammation and apoptosis (128, 129). Until now, the presence of four p38 family members has been identified: p38α, p38β, p38γ and p38δ (131). Of these, p38α and p38β are highly expressed in various tissues, but p38γ and p38δ are differentially expressed, depending on the type of tissue (132). The phosphorylation of p38 mediates multiple transcription factors such as ATF-1, ATF-2, Sap-1, Elk-1, NFκB, Ets-1 and p53, which mediate extracellular stress responses, inflammation and cell apoptosis (128, 129). Activation of these MAPK cascades (ERK1/2, JNK1/2 and p38) by GnRH treatment is dependent on the cellular context. GnRH treatment induced activation of ERK1/2 through PKC, c-Src, dynamin, Ras and Ca^{2+} signaling in αT3-1 and LβT2 cells (133). The phosphorylation of JNK1/2 by GnRH in αT3-1 cells is mediated by PKC, c-Src, CDC42/Rac1 and MEKK1 pathways

(134), while the activation of p38 by GnRH is mediated by a PKC-dependent cascade (135).

1.3. Ovarian granulosa cell lines

The studies for the regulation of granulosa cell function are commonly performed in primary cultured granulosa cells, because of their availability. However, primary cultured granulosa cells have some limitations such as limited proliferative capacities and life-span. Therefore, a numner of granulosa cell lines have been established from various species including human. However, as shown in Table 1, both primary cultured and immortalized granulosa cells have advantages and disadvantages.

1.3.1. Rodent granulosa cell lines

1.3.1.1. By oncogene transformation

Simian virus 40 (SV40) is one of polyomavirus, having a capability for transforming a number of cell types with two viral proteins: large and small T antigen, respectively. Large T antigen is a hexamer viral protein that is a one of virus-encoded proteins derived from two transcriptional units by differential RNA splicing. The transformation capabilities of SV40 Large T antigen are mostly mediated by the

functional inactivation of retinoblastoma (pRB) family members and/or p53 tumor suppressor proteins (136).

Through the use of SV40 large T antigen transfection, the GC48C cell line was the first granulosa cell line from rat developed to continually produce progesterone (137). This cell line exhibits progesterone production that is strongly responsive to cAMP (137). When compared with rat primary granulosa cell culture, this cell line releases a similar amount of steroid hormone. However, the GC48C cell line was not steroidogenically responsive to FSH. Using SV40 large T antigen transfection, a rat granulosa cell line, RGA-41S, was established. Granulosa cells were isolated from 27 day old immature rats (138). The RGA-41S cell line showed normal proliferation and transformed morphology at 33 °C but displayed restricted proliferation potential and non-transformed morphology at 40 °C. By v-myc transfection, three granulosa cell lines (GRM01, GRM02, and GRM01L) were established from PMSG-stimulated mice (139).

1.3.1.2. From tumors in transgenic mice

A granulosa tumor cell line, KK-1, has been established by SV40 T-antigen transfection of tumor tissue (140). However, the KK-1 cell line displayed minimal

steroidogenic responsiveness to FSH. The steroidogenic responsiveness to LH and FSH treatment was maintained until passage 10 and 20, respectively.

1.3.1.3. By radiation

A granulosa tumor cell line, OV3121, was established by irradiation with gamma rays from B6C3F1 mice (141). The production of estradiol was measured, but the production of progesterone and estriol was not detected. Responsiveness to cAMP and gonadotropin in this cell line was not determined.

1.3.1.4. By chemical treatment

An ROG cell line was established by chemically mediated mutagenesis from immature rat. An ROG cell line was established by treatment of serum-free granulosa cell culture with a chemical mutagen (142). This cell line has been grown continuously with recombinant activin A, known to increase granulosa cell growth and stimulate FSH receptor expression (143-145). The cell line displayed FSH-mediated steroidogenesis and cell proliferation.

1.3.1.5. By spontaneous immortalization

A spontaneously immortalized cell line, SIGC, was generated from rats (146). This cell line displayed typical epithelial morphology and continuous proliferation without the morphological characteristics of luteinization (146). SIGC cells displayed undifferentiated characteristics such as prominent gap junctions, desmosomes and intermediate filaments (146). The SIGC cell line produced a small amount of progesterone and estrogen. Levels of responsiveness to cAMP, gonadotropins and forskolin were not determined in this cell line. Serum starvation or bFGF treatment induced apoptosis in the SIGC cell line (147).

1.3.2. Porcine granulosa cell lines

MDG2.1 cell line was established by SV40 T antigen transfection of primary cultured granulosa cells from porcine follicles (148). The expression of steroidogenic enzymes, such as CYP11A, was identified in this cell line, but the steroidogenic potential of the MDG2.1 cell line has not been determined. This cell line expressed IGF-I and IGFBP.

The PGC-2 cell line was established by continuous sub-culture of primary cultured porcine granulosa cells with horse chorionic gonadotropin (149). This cell line

displays great proliferative potential. This cell line was not steroidogenically responsive to FSH and LH, but progesterone production was highly increased with the addition of progesterone precursor (pregnenolone).

Another porcine granulosa cell line, jc-410, was spontaneously established (150, 151). The Jc-410 cell line was established from granulosa cells derived from follicles that were 4-6 mm in size, and this cell line showed limited progesterone production potential when compared to primary cultured granulosa cells (150). The production of estradiol was only detected with the addition of precursor (androstenedione). The Jc-410 cell line did not show responsiveness to LH or FSH, but displayed responsiveness to cAMP and forskolin in a dose-dependent manner (151).

1.3.3. Bovine granulosa cell lines

A spontaneously immortalized bovine granulosa cell line (BGC-1) was established through continuous in vitro sub-culturing after passage 75 (152, 153). This cell line displayed quite limited progesterone production potential, even with the addition of cAMP. The BGC-1 cell line produced a small amount of progesterone following treatment with pregnenolone as a precursor, indicating lower functional activity of 3B-HSD in this cell line (152, 153). This cell line did not show any

steroidogenic responsiveness to cAMP, and gonadotropin responsiveness was not determined.

1.3.4. Primate ovarian granulosa cell lines

A G1SV1 cell line was established by SV40 T antigen transfection of primary cultured granulosa cells from preovulatory follicles derived from marmoset monkey (154). Basal potential for progesterone synthesis was very limited, but treatment with cAMP significantly increased progesterone production (154). The expression of ER-α mRNA was detected in this cell line, and expression was significantly decreased by cAMP treatment. PR mRNA expression was not detected in the presence of cAMP. The expression of 3B-HSD was demonstrated by immunocytochemistry. However, gonadotropin responsiveness was not determined in this cell line (154).

1.3.5. Human ovarian granulosa cell lines

1.3.5.1. From ovarian tumors

1.3.5.1.1. HTOG cell line

The first granulosa cell tumor cell line was isolated from a granulosa-theca cell tumor in a Japanese woman (155). This tumor cell line showed a high degree of

chromosomal abnormality. This cell line displayed no contact inhibition of growth and was able to grow into multiple cell layers. The HTOG cell line produced significant amounts of estrone and estradiol, but limited amounts of progesterone and 17-hydroxyprogesterone, but no detectable level of testosterone (155). However, gonadotropin responsiveness was not determined in this cell line.

1.3.5.1.2. COV 434 cell line

Metastatic granulosa tumor cells were derived by biopsy of a female patient with a granulosa cell tumor of the ovary (156, 157). The isolated granulosa tumor cells displayed the typical characteristics of luteinized granulosa cells. This granulosa tumor cell line produced a significant amount of estradiol in the presence of FSH and androstenedione (157). Treatment with LH and hCG did not induce a detectable amount of estradiol production, and the expression of LH receptor mRNA was not identified in this cell line. Although cAMP synthesis was increased by treatment with FSH and forskolin, progesterone production was not detected following gonadotropin treatment (157). This cell line is the first established gonadotropin-responsive granulosa tumor cell line.

1.3.5.1.3. KGN cell line

The KGN cell line was obtained from a female Japanese patient with invasive Stage III granulosa cell carcinoma (158). The cells grew as a monolayer for more than 100 passages. The expression of CYP19 enzyme was detected by immunohistochemical analysis. Following treatment with cAMP, the production of progesterone and pregnenolone was increased in a dose-dependent manner (158). The production of 17α-hydroxyprogesterone and 17α-hydroxypregnenolone was not detectable following cAMP treatment (158). CYP17 mRNA was not detected by real-time PCR in the KGN cell line.

1.3.5.2. By oncogenic transformation

1.3.5.2.1. HGL5 cell line

An immortalized human granulosa cell line (HGL5) was established by transfection with E6 and E7, which are parts of human papillomavirus 16 (HPV16) (159). The HGL5 cell line showed increased proliferation potential. A basal level of progesterone production was detected, but treatment with gonadotropins (LH or FSH) did not affect progesterone production in this cell line (159). Increased progesterone production was detected following forskolin or cAMP treatment. The expression of

CYP19 in this cell line was lower than that of a primary granulosa cell culture (159).

1.3.5.2.2. HO-23 cell line

The HO-23 cell line was established by transfection with SV40 large T antigen together with tumor suppressor gene p53 and Ha-ras oncogene (160). Treatment with forskolin significantly increased production of progesterone and pregnenolone compared to basal levels, in this cell line (160). Although progesterone production was significantly increased by the treatment with cAMP, progesterone production was not detected following hCG treatment. The expression of StAR was markedly enhanced by forskolin treatment in this cell line (160).

1.3.5.2.3. GC1a cell line

A GC1a cell line was established by transfection with SV40 large T antigen (161, 162). Granulosa cells were isolated from 6-mm follicles from Japanese patients undergoing oophorectomy and hysterectomy (161). The GC1a cell line showed highly increased proliferation potential and epithelial-like morphology (161). The production of progesterone was not detected following gonadotropin stimulation in this cell line (162). The cells did not synthesize progesterone but did synthesize estradiol. The rate

of estradiol synthesis was not altered by androstenedione treatment in this cell line, but it was increased by estrone treatment (162). Responsiveness to gonadotropin, cAMP and forskolin was not determined in this cell line.

1.3.5.2.3. SVOGs cell line

SVOGs cell lines were established by transfection with SV40 large T antigen (163). Primary cultured human granulosa cells were isolated from patiens undergoing IVF therapy. The GC1a cell line showed increased proliferation potential and epithelial, elongated, and fibroblastlike orphology. SVOGs cell lines responded to hCG, cyclic AMP and pregnenolone, not to FSH(163).

1.4. Granulosa cell apoptosis

The apoptosis of ovarian granulosa cells is complexly regulated by several pro-apoptotic and anti-apoptotic signaling molecules (164). A variety of factors are involved in the regulation of granulosa cell apoptosis: a) the B cell/lymphoma-2 family (Bcl-2) pathway; b) the TNF-α signaling pathway; c) the caspase signaling pathway and d) the protease-apoptosis activating factor-1 signaling pathway.

1.4.1. Bcl-2 family

The Bcl-2 family comprises two sub-families: anti-apoptotic members (Bcl-2, Bcl-XL, Mcl-1 and A1) and pro-apoptotic members (Bax, Bak, Bad, Bim, Bid and Bik) (165). The activity of Bcl-2 family proteins is determined by translational as well as post-translational modifications, including transcription, proteolytic-cleavage, dimerization, translocation and phosphorylation (166). The regulation of Bcl-2 mRNA expression can be an important determinant in the control of cell apoptosis. For example, an enhanced expression level of Bcl-2 protein in tumor cells has been suggested (167). Because of their ability to form hetero- or homodimers, Bcl-2 family proteins can regulate cell death alone or in combination with other protein family members (168). The phosphorylation status of Bad can modulate its intracellular location and its ability to engage in protein-protein interactions. Survival signals significantly induce phosphorylation of two amino acid sites on Bad. Phosphorylated Bad binds to 14-3-3 regulatory protein and is then sequestered in the cytoplasm (169). Bid is a relatively small protein with a long flexible loop domain in the cytosol that mediates a survival signal. Caspase-8 is activated and mediates the cleavage of the long flexible loop of Bid by stimulation of apoptoric signaling, causing mitochondrial dysfunction and apoptosis (170). The homodimerization of Bax and Bax protein is

induced in the presence of apoptotic signaling, and this homodimeric protein is translocated from the cytosol to the mitochondrial membrane, causing mitochondrial dysfunction and apoptosis (171). The expression of Bcl-2 and Bax is detected in granulosa cells of both fetal and adult ovaries (172). High levels of Bcl-2 are found in healthy developing follicles, and strong Bax expression is found in apoptotic or atretic follicles (173). The expression of Bcl-2 is correlated to gonadotropin expression level; gonadotropin inhibits Bax expression and induces Bcl-2 expression in human corpus luteum (174). The functional effects of Bcl-2 family members in granulosa cell apoptosis are demonstrated by: a) increased numbers of atretic follicles in bcl-2 (-/-) mice (175); b) inhibition of granulosa cell apoptosis mediated by enhanced expression of Bcl-2 protein (176, 177); c) infertility due to follicles with excess granulosa cells in Bax (-/-) mice (166); d) follicles that are atretic rather than growing due to high levels of Bax expression (178). Other Bcl-2 family proteins including BAD, Bok and Mcl-1 also mediate granulosa cell apoptosis (179, 180).

1.4.2. The tumor necrosis factor (TNF) family

TNF initiates its functional activity by binding to receptors (TNF-receptors I or II) and activating multiple signaling pathways (181). TNF receptor-I contains a

death domain (DD) motif within the intracellular region, whereas TNF receptor-II does not contain a death domain (DD) motif (181). The trimerization of the death domain (DD) in TNF receptor-I is caused by the binding of TNF-α in most human tissues (182). TNF-receptor-I recruits an adaptor protein, TNF receptor-associated death domain (TRADD). The TRADD may then recruit an additional adaptor protein, the Fas-associated death domain (FADD) (182). FADD functions as a platform adaptor that recruits procaspase-8 through direct protein interaction to form a death-inducing signal complex (183). TNF receptor-II does not contain a cytosolic death domain (DD). Therefore, the interaction between TNF- α and TNF-receptor-II results in the recruitment of TRAF-1 and TRAF-2 (184). TNF-receptor-II does not have a death domain (DD) but may facilitate TNF-Receptor-I-mediated apoptosis by increasing levels of membrane-associated TNF-α. TRAF-2 serves as an adaptor protein that mediates the interaction of TRAF-2 and RIP (184). The interaction between TRAF-2 and RIP results in the activation of nuclear factor kappa B (NFκB) (185). TNF family members such as TNF-α, Fas/Fas-ligand (FasL) and TRAIL are known to be involved in follicular development and apoptosis (186). TNF-α is one of the important regulators of granulosa cell apoptosis in many species including human (187-189). Most of the biological functions of TNF-α are mediated through TNF receptor-I, which contains a

death domain (DD) within its intracellular region (190). Expression of TNF receptor-I has been identified in ovarian granulosa cells of many species including cow, pig and chicken (191-193). A Fas (also known as CD95) is an apoptosis-mediating receptor, and its ligand (Fas Ligand) mediates apoptosis by binding to its receptor (Fas). The expression of Fas and Fas ligand has been demonstrated in rat, cow and human (194-196). In a porcine model, the expression of Fas ligand was lower in granulosa cells from large follicles than in those from small follicles, and serum deprivation increased expression of both Fas and Fas ligand in a time-dependent manner (197). The functional roles of Fas/Fas-ligand have been demonstrated by several researchers: a) the level of Fas ligand was highest in theca and granulosa cells from subordinate apoptotic follicles as compared with healthy growing follicles (198); b) the Fas/Fas ligand system mediated interferon-gamma induced granulosa cell apoptosis in rat (199). Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is one member of the TNF superfamily that induced apoptotic cell death in various cancer cells (200, 201). When TRAIL binds to its receptor, the trimerizations of TRAIL receptor-I or II occurs, and then multiple apoptotic signaling pathways are activated (200, 201). The functional roles of TRAIL/TRAIL receptor have also been demonstrated by several researchers: a) the expression of TRAIL and its receptors has been established in

human granulosa cells (202); b) TRAIL also mediated cisplatin-induced apoptosis in human granulosa tumor cells (203).

1.4.3. Caspases

The caspase family is a family of cysteine proteases that can mediate multiple apoptotic pathways by their ability to cut other target proteins after an aspartic acid residue (204). Caspases choose their target substrate by recognizing a four-amino-acid residue including an aspartic acid that serves as the target sequence for the cleavage reaction (204). The caspase family members overlap in terms of substrates and target cleavage motifs (205). A particular four-amino-acid substrate can be shown to be a "caspase-4 substrate" or a "caspase-7 substrate", but this does not mean that a caspase-4 substrate can only be cleaved by caspase-4 (205). However, a caspase-4 substrate is most efficiently cleaved by caspase-4. It has been reported that caspase family members share common structure and substrate specificity. Caspase is expressed as an inactive pro-enzyme with an NH_2-terminal domain, a large and a small subunit (206). The NH_2-terminal domain of caspase is highly variable in terms of sequence and is involved in the regulation of enzyme activity (206). The proemzyme is proteolytically cleaved to produce an active enzyme (206). Caspase is one of the important regulatory

molecules in granulosa cell apoptosis. The expression of caspase-1 and caspase-3 has been demonstrated in granulosa cells from *in vitro* fertilization (IVF) patients (207). GnRH-I or -II induce apoptosis through a proteolytic caspase cascade including caspase-8 (the initiator) and caspase-3 and -7 (the effectors) in human granulosa cells (208). Caspase-3-dependent apoptosis occurs in human granulosa cells during atresia and luteinization (209). The expression of caspase-3 is significantly decreased in healthy growing follicles, and expression is regulated by gonadotropin level in granulosa cells (210).

1.4.4. Apoptotic protease-activating factor 1 (Apaf-1)

Without apoptotic stimuli, mitochondrial intermembrane-space proteins such as cytochrome c are isolated in the inter-membrane spaces of mitochondria (211). A number of apoptotic stimuli cause the release of large molecules such as cytochrome c from the inner membranes of mitochondria to cytoplasm, where they trigger a subsequent cytochrome c-dependent-apoptotic cascade (211). The released Cytochrome c from mitochondria binds to Apaf-1 (apoptosis activation factor 1), which is expressed in the cytoplasm and contributes to the induction of apoptosis by facilitating the formation of the apoptosomal complex (211). The resulting complex

mediates proteolytic cleavage of procaspase-9 in the cytoplasm (212). Activated caspase-9 then initiates the down-stream caspase-dependent apoptotic pathway, leading to cell death. In mice, gonadotropins treatment significantly inhibited the expression of Apaf-1 as well as apoptosis in granulosa cells (213).

Table 1 Advantage and disadvantage of the primary cultured and immortalized human granulosa cells.

Cell types	Advantage	Disadvantage
Primary cultured cell	1) Close to normal morphology and function.	1) Limited cell number 2) Limited life span in vitro 3) Heterogeneous population 4) Under stimulation with high dose of hormones 5) Different clinical history
Cell lines	1) Simple to culture 2) Extended life span 3) Large number of cells 4) Possible to cryopreserve 5) Homogeneous population	1) Change cell morphology, function or the expression of genes by oncogene transfetion. 2) Lack of representativeness

2. Hypothesis and objectives

2.1. Rationale

During follicle development, ovarian granulosa cells display a high degree of structural changes and secrete hormones that allow not just endocrine signaling to target tissues, but also paracrine, and autocrine signaling to adjoining granulosa cells, as well as growth and maturation of the oocyte. Although some of the molecular features of granulosa cells have been characterized, these studies are usually confined to animals (214-217) and granulosa tumor cells (218, 219); alternatively, only one or a few of these features have been studied in normal human granulosa cells (220-222). Thus, a combined profile providing a more comprehensive appreciation of the human granulosa cell is lacking.

GnRH is the central hypothalamic regulator of reproductive function (223, 224). In addition to its well-known regulatory roles in the hypothalamus-pituitary-gonadal axis, the expression of GnRH and GnRH receptors has been established in human reproductive tissues such as the ovary (225), placenta (226), endometrium (99), and testis (227). The expression of GnRH receptors has also been reported in human ovarian granulosa cells (71, 77, 228). GnRH has been shown to have anti-proliferative effects on granulosa cells from rats (229, 230), pigs (78, 231) and humans (83, 232), but the underlying mechanisms remain unclear.

2.2. Hypothesis

Collectively, these observations suggest that the functional roles and molecular mechanisms of GnRH-I or II in human granulosa cells should be investigated. As GnRH and it's receptor present in human ovarian cells and have functional roles via activating multiple signaling pathway, GnRH system may play a key role during follicle development and atresia. Therefore, we have hypothesized that GnRH-I, II and it's receptor play important roles in human follicule development and atresia.

2.3. Objectives

Specific aim 1: To investigate multiple molecular characteristics of human granulosa cells by analyzing the expression patterns of cytoskeletal proteins, cell adhesion molecules, steroidogenic enzymes, steroid receptors and gonadotropin receptors in human primary cultured granulosa cells. Although some of the molecular features of granulosa cells have been characterized, studies are usually confined to animals (214-217) and granulosa tumor cells (218, 219) or studied in a limited capacity in normal human granulosa cells (220-222). Thus, a combined molecular profile providing a more comprehensive appreciation of the human

granulosa cell is lacking.

Specific aim 2: To create new immortalized human granulosa cell lines so that a continuous supply is available as a study model to investigate human granulosa cell functions. To investigate some of the cellular and molecular characteristics of human granulosa cells, stable granulosa cell lines can be useful research tools. Therefore, the second objective of our study was the creation of new immortalized human granulosa cell lines.

Specific aim 3: To characterize multiple cellular and molecular features of these immortalized cells as compared to those of primary cultured granulosa cells. To determine whether newly established granulosa cell lines were equipped with properties considered essential for normal granulosa cell functions, we have characterized multiple cellular and molecular features of these immortalized cells for comparison with those of primary cultured granulosa cells - the expression patterns of cytoskeletal proteins, adhesion molecules, steroidogenic enzymes, steroid receptors, gonadotropin receptors, morphology, anchorage-independent growth, growth potential, and progesterone production.

Specific aim 4: To investigate the effects of GnRH-I or II on granulosa cells using immortalized human granulosa cells as the experimental tools. We used the TUNEL (terminal deoxynucleotidyl-transferase (TdT)-mediated biotin-dUTP nick-end labeling) assay to evaluate apoptosis induced by GnRH-I or GnRH-II.

Specific aim 5: To determine whether GnRH-I or II exert their apoptotic effects through activation of the caspase pathway in granulosa cells. Whether GnRH-I or -II can activate the caspase pathway in human granulosa cells is unknown. Therefore, western blotting was performed to investigate the role of the caspase signaling in GnRH-I- or II-induced apoptosis with specific antibodies for caspase-3, 7 and 8.

Specific aim 6: To examine the role of FSH on GnRH-I- or -II-mediated apoptosis in human granulosa cells. FSH is a key survival factor during human follicular development (233). In vitro, FSH prevents atresia of cultured rat preovulatory follicles (234) To investigate the anti-apoptotic effects of FSH, human immortalized granulosa cells were cultured with both FSH and GnRH-I or -II.

Specific aim 7: To investigate whether GnRH-I or -II induce apoptosis through a Bcl-2 family-dependent intrinsic pathway in human granulosa cells. Various studies have provided evidence that members of the Bcl-2 family are important regulators of follicle cell apoptosis (235, 236). Therefore, in the present study, we investigated the effect of GnRH-I or -II on the expression and phosphorylation of several Bcl-2 isoforms.

Specific aim 8: To investigate the attenuating effects of GnRH-I or -II on IGF-I activity. IGF-I can stimulate human granulosa cell proliferation (237) and increase steroid secretion and responsiveness to gonadotropins in ovarian follicles of sheep (33, 238), cows (33, 239) and humans (240). Recent studies also suggest that GnRH agonists can interfere with IGF-I receptor expression and activation in androgen-independent human prostate cancer cells (241) and mouse pituitary cells (242). There is currently no information on whether GnRH-I or -II can affect the IGF-I activities in human ovarian granulosa cells.

Specific aim 9: To determine whether the stimulatory effects of GnRH-I or -II on granulosa cell apoptosis were mediated through gap junctional communication in

human granulosa cells. It has been demonstrated that gap junctional communication plays prominent roles in human granulosa cell differentiation, development and survival (243-245). GnRH analogs can induce phosphorylation of Cx43 and reduce Cx43 mRNA expression in rat granulosa cells (246). However, the effects of GnRH-I or -II on gap junctions and Cx43 in human granulosa cells are unknown.

3. Molecular characterization of human granulosa cell and establishment of immortalized human granulosa cell lines

3.1. Introduction

Although some of the molecular features of granulosa cells have been characterized, they are usually confined to animals (214-217) and granulosa tumor cells (218, 219), or studied with one or a few of these features at a time in normal human granulosa cells (220-222). Thus, a combined profile providing a more comprehensive appreciation of the human granulosa cell is lacking.

Vimentin plays important roles in anchoring the position of the organelles in the cytosol (247), maintaining cell integrity (248) and transporting of low-density lipoprotein (249). Cytokeratins associate between the plasma membrane and the nuclear surface providing important implications for dynamic cellular processes such as mitosis (250), movement (251) and differentiation (252). Desmin maintains the structural integrity of the cell (253, 254) and may be involved in mitochondrial function (255). The expression of these cytoskeletal proteins is organ or tissue specific. As the human follicle matures, cytokeratin appears to decrease, whereas vimentin remains unchanged (256). Desmin expression is minimal or absent in human granulosa

tumors cells (257) but its expression in normal human granulosa cells remains to be elucidated.

High Cx43 levels have been linked to good quality oocytes and follicular growth in human luteal-granulosa cells (258). The initiation of luteinization leads to increased E-cadherin expression in rat luteinizing follicle (259) but its expression in human granulosa cells is unknown.

StAR (260), P450scc (261) and 3β-HSD (262) are important elements in steroidogenesis and markedly expressed in human luteal-granulosa cells.

The expression of ER-α and β was decreased by GnRH agonist and hCG in human luteal-granulosa cells (220). There were no differences in PR mRNA expressions at different stages of the luteal phase in human corpus luteum (263) but the detailed expression of each isoform of PR protein (α, β) in human granulosa cells uncertain.

LH receptor remained low in the late follicular phase and elevated in the mid-luteal phase, whereas FSH receptors were at a high level in the late follicular phase and decreased in the mid-luteal phase (264). However, their detailed expressions in human unluteinized granulosa cells remain to be elucidated. Therefore, the first objective of this study was to investigate multiple characteristics concurrently to obtain a better

knowledge about human granulosa cells.

The regulation of granulosa cell functions is commonly investigated by primary cultured granulosa cells obtained from in vitro fertilization (IVF) procedures because of their availability. However, human granulosa cells isolated from IVF procedures have limited proliferative capacity and can rarely be cultured for longer than a few days. These cells are usually pooled for culture and are therefore not homogeneous (150), and consequently, experimental results can vary between studies.

To investigate some of the molecular characteristics of human granulosa cells, stable granulosa cell lines are additional research tools. Using SV40 large T antigen, Lie *et al* (163) established a number of immortalized human granulosa cell lines, collectively named SV40 transformed ovarian granulosa cell lines (SVOGs)**.** While SVOGs acquire an increased growth potential to allow further subcultures, supplies of these original cell lines are no longer available for research.

Therefore, the second objective of this study was two-fold: 1) to create new human granulosa cell lines so that a continuous supply is available as study models for granulosa cell functions; 2) to take the opportunity to characterize multiple features of these immortalized cell lines and compare with those of primary cultured granulosa cells identified from our first objective.

3.2. Materials and methods

3.2.1. Granulosa cell culture

Primary cultured human granulosa cells were collected from the University of British Columbia IVF Program. Isolation and primary culture of granulosa cells were modified from the work of Lie *et al* (163). The cell suspension was gently spin in a centrifuge at 500×*g* for 10min, and the supernatant removed. The final pellet was resuspended in 5 ml of M-199/MCDB 105 (1:1 mixture) with 10% FBS. Granulosa cells can be separated from other cell types by centrifuging for 10 min at 2800*g* on a single density Percoll layer. Isolated granulosa cells were then cultured in M-199/MCDB 105 (1:1 mixture) with 10% FBS and maintained at 37°C in humidified atmosphere of 5% CO_2 in air.

3.2.2. Reagents

Medium 199 (M-199), MCDB 105 medium (MCDB 105), 8-bromoadenosine 3`-, 5`- cyclic monophosphate (8-br-cAMP) and fetal bovine serum (FBS) were purchased from GIBCO RRL (Grand island, NY, USA). FuGENE 6 transfection reagent was purchased from Roche Co. (Roche Molecular Biochemicals, Germany). Anti-SV40 antibody, anti-vimentin antibody, anti-cytokerain 5/6 antibody, anti-desmin

antibody, anti-3β-HDS antibody, anti-P450scc antibody, anti-StAR antibody, anti-ER-α antibody, anti-ER-β antibody and anti-PR antibody were purchased from Santa Cruz Biotechnology (CA, USA). Anti-connexin 43 antibody was purchased from Cell Signaling Technology, Inc. and anti-E-cadherin antibody was purchased from BD Transduction Laboratories (Heidelberg, Germany).

3.2.3. Western blot analysis

After each treatment, cells were washed two times with PBS and lyzed in RIPA buffer containing phosphatase inhibitor cocktail I (Sigma Chemical, St. Louis, MO). The protein content of the supernatants was determined by the Bio-Red DC protein assay (Bio-Rad Laboratories, Inc.). Samples fractionated on SDS-PAGE at constant voltage were then transferred onto nitrocellulose membranes for 2h. The membrane was blocked by incubation in 10ml PBS containing 5% skim-milk powder at room temperature for 1h. The membrane was washed in PBS containing 0.05% Tween 20 (Tween-PBS; Sigma Chemical, St. Louis, MO), and then incubated overnight with the specific primary antibodies at 4°C. Secondary antibodies diluted in the same solution were then added to each membrane, incubated for 1 h at 37°C, rinsed three times with

PBS containing 0.05% Tween 20. Each band on western blotting was quantitated with the Scion Image Software (Scion, Frederick, MD) using B-actin as the internal control.

3.2.4. Immunofluorescence analysis

Immunocytochemistry was performed to confirm the expression of the various target proteins in granulosa cells. Cells were plated in an 8-well chamber slide and fixed with 3.7% paraformaldehyde in PBS for 15 min at room temperature. The samples were then permeabilized with 100% ice cold methanol for 10 min, 100% acetone for 10 min and 0.5% Triton X-100 (Sigma) in PBS for 10 min, with three times PBS washing in between each treatment. The samples were then incubated with blocking solution for 1h to block non-specific binding at 37°C. Primary antibodies diluted in the same solution were then added to each well of a chamber slide, incubated for 1 h at 37°C, rinsed three times with PBS. Fluorescent secondary antibodies diluted with the same blocking solution were then added and incubated for 45 min at 37°C. Each slide was rinsed three times again with PBS and a coverslide was added after removing the chamber well. Fluorescence was visualized using a fluorescence microscope.

3.2.5. Real-time PCR

RNA was extracted from the cells using 1 mL of Trizol reagent (Invitrogen, Life Technologies, USA) according to the manufacturer's instructions. Total RNA was reverse-transcribed into cDNA using a first-strand cDNA synthesis kit (GE HealthcareBio-Science, Piscataway NJ, USA) following the manufacturer's procedure. The primers used for SYBR Green real-time RT-PCR were designed using Primer Express Software v2.0 (Applied Biosystems, Foster City, CA, USA). Gene specific primers for detetecting LH receptor (forward: 5'-ACACTTTATTCTTCCATGCTTGCTGAG-3' and backward: 5'-ATTAAAGCATCTGGTTCAGGAGCACA-3') and FSH receptor (forward: 5'-TGTATCTTATGGTACTGTAACTG-3' and backword: 5'-GGGCTAAATGACTTAGAGGGACAA-3') were used in the RT–PCR.

Data were reported using a common threshold, where the cycle threshold (Ct) value is defined as the fractional PCR cycle number that requires for the fluorescent signal to exceed the detection threshold (ie exceeds background level). The Ct level is used in data quantitation. Ct value is inversely proportional to the amount of template DNA in the sample, ie, the greater the amount of target DNA in the sample, the lower the Ct value. In addition, GAPDH specific primers (forward: 5'-

ATGGAAATCCCATCACCATCTT-3' and backward: 5'-CGCCCCACTTGATTTGG-3') were used in the RT–PCR to quality of cDNA template from each sample.

3.2.6. Transfection of SV40 large t antigen

Primary cultured granulosa cells were transfected by SV40 large T antigen using FuGENE 6 transfection reagent (Roche Molecular Biochemicals, Germany) according to the manufacturer's instructions. Briefly, 1.5 µl of plasmid and 40µl of FuGENE 6 in 3 ml of serum/antibiotic-free medium were added to the cell culture. After 12h of incubation at 37°C, fresh medium was added to the culture. A successful transfection was confirmed two weeks later by a sharp increase in cell doubling levels and SV40 large T antigen expression.

3.2.7. Analysis of growth potential

Extended lifespan was evaluated by serial sub-cultures and direct cell counting. In each subculture, cells were plated at 2 x 10^5 seeding densities and harvested using trypsin after culturing for 5 days. Viable and nonviable cells were detected by trypan blue and counted using a hemocytometer. The extended lifespan of transfected cells

was determined by their total cumulative population-doubling levels (CPDL) in continual subculture from a known number of cells. The CPDL in continual subculture was determined by calculating the following formula CPDL = ln(*Nf*/*Ni*)/ln 2, where *Ni* and *Nf* are the initial cell numbers and final cell numbers, respectively, and ln is the natural log.

3.2.8. Anchorage-independent growth assay

Anchorage-independent growth (AIG) capability of immortalized cell lines was determined by assessing the ability of colony formation in soft agar. Granulosa cells (1×10^4) suspended in culture medium containing 0.33% "soft" agarose (0.5% Type I, Low EEO; Sigma Chemical Co.) were overlaid on top of 0.5% "hard" agarose layer. Additional medium was added on the top and changed every 3 days. After culture for 4-weeks, the formation of colonies in the soft agar was determined by phase contrast microscopy.

3.2.9. Progesterone production

Progesterone (P4) concentration was determined by Progesterone Correlate-EIA™ kits which purchased from Assay Designs (Ann Arbor, MI). P4 levels were

assayed according to the manufacturer's instructions. Briefly, anti-P4 antibody, samples and 50 ul of HRP-conjugated progesterone were added to 96-well plate pre-coated with goat anti-mouse antibody and incubated overnight at 4°C. Substrate solution was applied to all wells and incubated for 30 min at 37℃. The color development on the plate was terminated by adding 50 ul of stopping solution to all wells and OD was estimated by measuring absorbance at 450 nm in an ELISA plate reader.

3.2.10. Statistical analysis

Results from more than three independent experiments were presented as the mean ± SD. Statistical analysis was carried out using GraphPad Prism 5.0. Data were analyzed by one-way analysis of variance (ANOVA) and non-parametric Kruskal–Wallis test. The level of significance was set at $p < 0.05$. Statistical significance was defined at a *P level of <0.05*.

3.3. Results

3.3.1. Characterization of primary cultured human granulosa cells

3.3.1.1. The expression patterns of cytoskeletal proteins

In this study, we identified the expression patterns of vimentin, cytokeratin 5/6 and desmin by immunocytochemical method and western blotting in primary cultured human granulosa cells. Vimentin and cytokeratin5/6 were well-expressed in all three patients studied whereas desmin was poorly expressed (Fig 1).

3.3.1.2. The expression patterns of cell adhesion molecules

As shown in Fig 2, Cx43 was densely localized in the cytoplasm as green dots in primary cultured human granulosa cells from all three patients. E-cadherin was also localized in the plasma membrane from all three patients, but the expression was weaker than that of Cx43 on western blot analysis. This weakly positive E-cadherin expression is consistent with corresponding low expression levels in unluteinized rat follicles (259).

3.3.1.3. The expression patterns of steroidogenic enzymes

As shown in Fig 3, StAR, P450scc and 3β -HSD were presented in granulosa

cells from all three patients consistent with previous observations from human luteal-granulosa cells of IVF patients, but biological variation exists as one patient (P2) had relatively lower expression of all three enzymes.

3.3.1.4. The expression patterns of steroid hormone receptors

As shown in Fig 4, the expression of ER-α, β and PR- α, β proteins in cultured human granulosa cells were investigated by western blot analysis and immunocytochemical analysis. The expressions of both ER-α and ER-β protein were detected in human primary cultured granulosa cells. Immunocytochemical analysis could not distinguish PR- α and β isoforms respectively; whereas western blot analysis revealed that the expression level of PR- α is higher than PR- β in granulosa cells.

3.3.1.5. LH receptor and FSH receptor mRNA expression

As shown in Fig 5, the mRNA of FSH receptor and LH receptor were both expressed in primary cultured human granulosa cells derived from three patients on real-time PCR. The relative expression of LH receptors was significantly higher than that of FSH receptors.

3.3.2. Transfection of human granulosa cells with SV40 large t antigen

After two weeks of culture in transfection agent, granulosa cells that were successfully transfected formed rapidly growing colonies, while untransfected granulosa cells displayed enlarged, flattened morphology and underwent senescence and degeneration. Although 18 colonies were obtained from all three patients, only 2 colonies from the one patient were able to bypass senescence. These two transfected colonies acquired extended lifespan and the cells exhibited an elongated shape (Fig. 6A), reaching confluence within a short period of time. The presence of SV40 large T antigen in transfected granulosa cells was confirmed in the cell nuclei by immunostaining and western blotting (Fig. 6B).

3.3.3. Analysis of growth potential

The extended lifespan of immortalized human granulosa cell lines was estimated by measuring the total CPDL (cumulative population doubling level) using the formula $\mathrm{CPDL} = \ln(Nf/Ni)/\ln 2$, where *Ni* and *Nf* are the initial cell numbers and final cell numbers, respectively, and ln is the natural log. The initial cell number was 2×10^5 for each subculture (265). The number of CPDL indicates the number of actual cell divisions. We then added the population doubling increase to the previous

population doubling level to obtain CPDL. Initially, 18 clones were derived from all three patients. Sixteen clones were not able to bypass senescence before confluence, but two clones derived from one patient were able to bypass senescence and acquire an extended lifespan (grew more than 30 CPDL), as shown in Fig. 7. Therefore, two SV40 large T antigen-transfected human granulosa cells acquired extended lifespans and appeared to be immortalized.

3.3.4. Anchorage independent growth

Normal animal cells require adhesion to a substratum in order to proliferate (266). Anchorage-independent growth is one of the characteristics of tumorigenic conversion of human cells in vitro (265). As a positive control for this set of experiments, we used cells from the ovarian carcinoma cell line, SKOV-3. After culture for 4 weeks, SKOV-3 cells formed large, anchorage-independent colonies in the soft agar, but the primary cultured granulosa cells did not. Representative microscopic photographs are presented in Fig. 8. In particular, the transfected cells from these two colonies (IHG1 and IHG2) that subsequently immortalized did not exhibit the anchorage-independent growth characteristics of tumor cells (Fig 8).

3.3.5. Characterizations of immortalized human granulosa cell lines

Western blotting analysis and real-time PCR were performed to investigate the expression patterns of cytoskeletal proteins (vimentin, cytokeratin5/6 and desmin), cell adhesion molecules (E-cadherin and connexin43), steroidogenic enzymes (Star, P450scc and 3β-HSD), steroid receptors (ER-α, ER-β, PR-α, and PR-β) and gonadotropin receptors (LH and FSH receptor) of two immortalized granulosa cell lines (IHG1 and -2), then compared with the results obtained using primary cultured granulosa cells.

As shown in Fig. 9, vimentin and cytokeratin 5/6 were expressed well, while desmin was poorly expressed in immortalized granulosa cells, which was consistent with results from primary cultured granulosa cells. However, levels apeared lower when compared with the results of primary cultured granulosa cells.

Cx43 and E-cadherin were also expressed in the two immortalized cell lines but the former had lower levels and the latter higher levels than the corresponding results for primary cultured granulosa cell cultures.

As shown in Fig. 10, ER-α and PR-β were strongly expressed in both immortalized cell lines, while these were poorly expressed in primary cultured granulosa cells. The expression of 3β-HSD was positive in both primary cultured and

immortalized granulosa cell lines. However, StAR and P450scc were poorly expressed when we compared our findings with results from primary cultured granulosa cells.

ER-β was expressed in both primary cultured and immortalized granulosa cell lines. However, the expression level of PR-α in two immortalized cell lines was lower than the corresponding result for primary cultured cells.

As shown in Fig. 11, the mRNA level of LH (P= 0.001, one-way ANOVA) and FSH (P= 0.0022, one-way ANOVA) receptor in the primary cultured human granulosa cells was higher than that of two immortalized granulosa cell lines. Non-parametric Kruskal–Wallis test reveal no significant difference between IHG2 and primary cultured granulosa cells for the mRNA level of LH receptor. Similarly for the the mRNA level of FSH, non-parametric Kruskal–Wallis test reveal no significant difference between IHG1 and primary cultured granulosa cells.

3.3.6. Evaluation of endocrine function

To determine whether immortalized cell lines (IHG1 and -2) retained steroidogenic potential for progesterone secretion, the immortalized cell lines were treated with LH, FSH and 8-Br-cAMP, as shown in Fig 12. In IHG1, an increase in progesterone production was obtained after 24 h incubation in the presence of cAMP

(P=0.0358, non-parametric Kruskal–Wallis test and P= 0.0134, one-way ANOVA). However, no significant difference in progesterone production was found after treatment with LH and FSH in both cell lines. Even though immortalized cell lines did not respond to stimulation by LH and FSH, they exhibited responsiveness to cAMP stimulation. Therefore, newly immortalized granulosa cell lines retain steroidogenic functions such as basal and/or cAMP -stimulated progesterone production.

3.4. Discussion

We have investigated combined multi-molecular profiles that play important roles in structures and functions of granulosa cells to elucidate more clearly the characteristics of human granulosa cells.

According to results from human tissues, as the follicle matures, cytokeratin content appears to decrease, whereas vimentin remains unchanged (256). Immunohistochemical localization of desmin was restricted to the theca layer in bovine ovaries (267) and also not expressed in the sheep secondary and tertiary follicles (268). However, the expression profile of desmin in human granulosa cells is still unclear. The expressions of vimentin and cytokeratin5/6 in our primary cultured granulosa cells were consistent with previous studies from human (256). Desmin was poorly

expressed in primary cultured human granulosa cells, similar to the sheep follicles.

In human granulosa cells from IVF patients, high cx43 (major gap junctional protein) levels have been linked to good quality oocytes (258), follicular growth, and expansion of the cumulus (269). Cx43 was strongly positive in primary cultured human granulosa cells from all three patients, the same as in a previous study from stimulated human follicles (258). The most prominent adhesion molecule of adheren junctions is E-cadherin. However, there is limited information about the expression profile of E-cadherin in human granulosa cells. The initiation of luteinization leads to increased E-cadherin expression in rat luteinizing follicle (259). It has been hypothesized that E-cadherin is a biomarker for granulosa cell luteinization. These weekly positive E-cadherin in primary cultured granulosa cells was consistent with corresponding low expression levels in unluteinized rat follicles (259).

According to previous studies on the expression of steroidogenic enzymes in rat and bovine, StAR (270) and P450scc (27) expressions are enhanced by the gonadotropin surge and their expressions are maintained at high levels in luteal phase (271). 3β-HSD is absent in the granulosa layer of most species until late follicle development (272). StAR, P450scc and 3β -HSD were well expressed in primary cultured granulosa cells from two patients but the expressions varied within the same

patient and also differed among the three.

In vitro expression profiles of ER- α, β (220) and PR- α, β (222, 263) have been reported in previous studies, which were conducted with human luteal-granulosa cells collected during IVF procedures. In our study, ER-β and PR-α were well expressed in primary cultured human granulosa cells from all three patients, whereas ER-α and PR-β were poorly positive. It is therefore quite possible that ER-β and PR-α are major steroid hormone receptors in human granulosa cells.

According to a previous study, LH receptor mRNA was increased by FSH treatment in a time-dependent manner in rat granulosa cells (273) and treatment with hCG resulted in a decrease or complete suppression of FSH receptor mRNA(274). FSH receptors were at a high level in the late follicular phase but decreased in the luteal phase in human (264). In contrast, LH receptors are induced in rat granulosa cells at the preovulatory stage by the actions of FSH and steroids (84, 275). LH receptor expression was also higher in granulosa cells of poor responders with prior IVF failure, compared to granulosa cells of normal responders (276). Therefore, it is possible that lower FSH receptor expression in primary cultured granulosa cells may result from the normal cyclic changes, the treatment with high doses of FSH and hCG during IVF cycles and/or that primary granulosa cells cultures were derived from low

responders who have lower LH receptors.

It is not surprising to find inter-patient biological variations of multi-molecular profiles during the characterization of primary cultured granulosa cells, because these variations reflect different clinical history, different treatments dose and/or agonists used during IVF cycles.

We also have established and characterized two immortalized granulosa cell lines (IHG1 and 2) by the transfection of SV40 large T antigen. Immortalized granulosa cell line has been already reported by several studies (159, 160, 162). Immortalized granulosa cell line has been also reported and has been used in our previous studies, including Hong et al (208). However, they only have reported morphological features of immortalized cells, their capability of progesterone production as well as their expressions of some stroidogenic enzymes with existing methodology.

In the two immortalized granulosa cell lines (SVOGs) created in this study, we investigated multiple characteristics that may be involved in the normal physiology and function of granulosa cells. Our new characterization methods have demonstrated that various essential properties considered for normal granulosa cells are present in our newly established cell lines. Firstly, newly established cell lines have steroidogenic

function such as both basal and cAMP-stimulated progesterone production.

Secondly, newly immortalized granulosa cell lines did not show anchorage-independent growth. Anchorage-independent growth is one of the most important characteristics for tumorigenic cell lines *in vitro*. These results indicate that even though newly immortalized granulosa cell lines have an extended lifespan, they have normal anchorage-dependent characteristics.

Thirdly, base on molecular characteristics from primary cultured granulosa cells, newly immortalized granulosa cell lines were characterized by comparing their molecular characteristics. Although there were some differential expression patterns of several molecular profiles, newly immortalized IHG1 and 2 were similar with that of primary cultured granulosa cells. It may be possible to interpret that these unexpected down or up- regulations of some characteristics are due to the influences of SV40 large T antigen (277, 278). Additionally, primary cultured granulosa cell populations obtained from different follicles during IVF procedure are heterogeneous. Therefore, it is also quite possible that newly immortalized granulosa cell lines were derived from some clones which have different characteristics from our primary cultures.

Collectively, although there were some biological variations from different patients, well-established characteristics of human granulosa cells are seem to be

helpful for better understanding of follicular functions and developments. Additionally, we have established two granulosa cell lines and determined their characteristics. Our well-characterized cell line systems can be a valuable source to study the potential mechanisms of follicular development and differentiation in human ovarian follicles in the future.

3.5. Figures

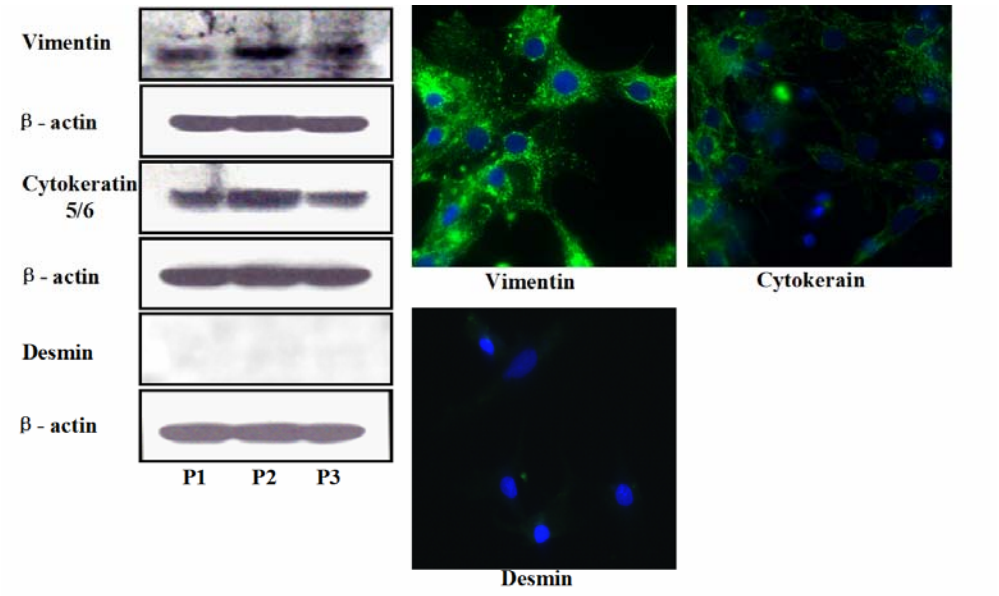

Figure 1. The expression patterns of cytoskeletal proteins in primary cultured human granulosa cells. After preculture for 24h, proteins were isolated from primary granulosa cell cultures of three patients (designated by P1, 2, and 3). The expressions of cytoskeletal proteins (vimentin, cytokeratin 5/6 and desmin) were assessed by western blotting and immunocytochemistry. Blue (DAPI) staining identifies cell nuclei. β-actin was the internal control.

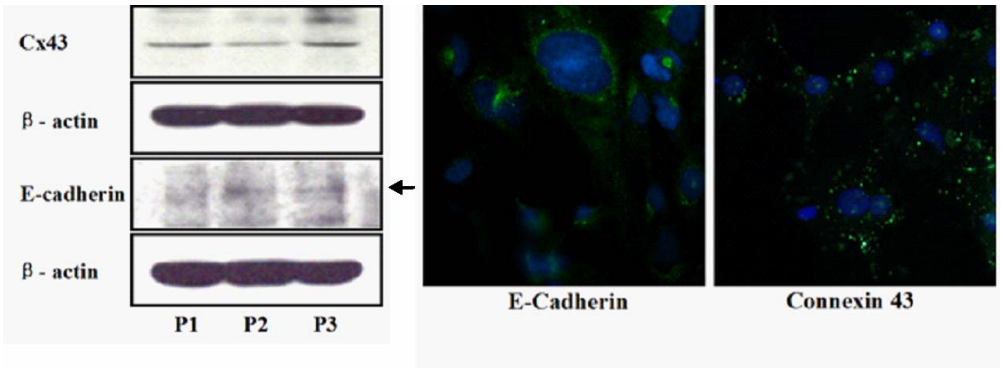

Figure 2. The expression patterns of cell adhesion molecules in primary cultured human granulosa cells. After preculture for 24h, proteins were isolated from primary granulosa cell cultures of three patients (designated by P1, 2, and 3). The expressions of adhesion molecules (Cx43 and E-cadherin) were assessed by western blotting and immunocytochemistry. Blue (DAPI) staining identifies cell nuclei. β-actin was the internal control.

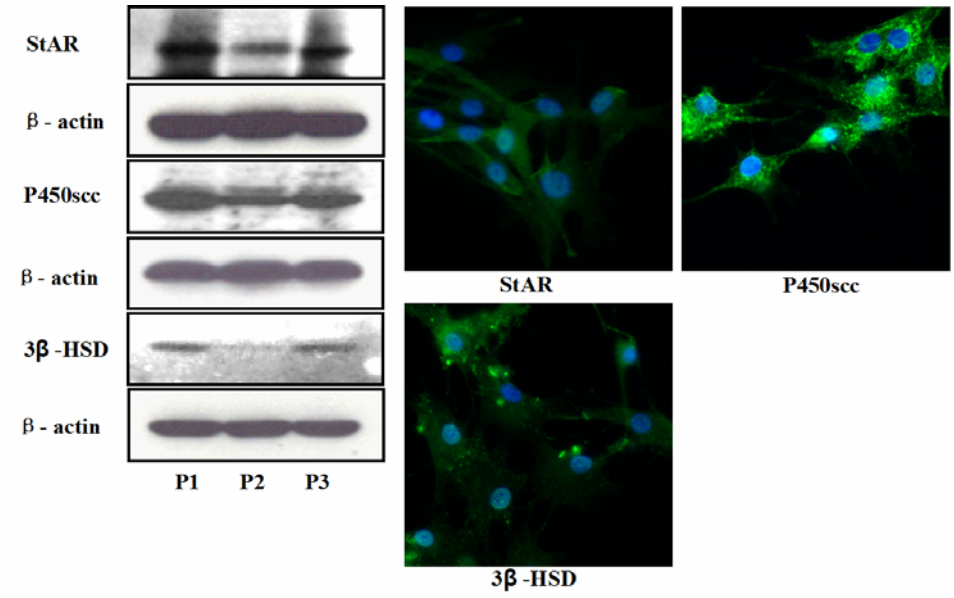

Figure 3. The expression patterns of steroidogenic enzymes in primary cultured human granulosa cells. After preculture for 24h, proteins were isolated from primary granulosa cell cultures of three patients (designated by P1, 2, and 3). The expressions of steroidogenic enzymes (StAR, P450scc and 3β–HSD) were assessed by western blotting and immunocytochemistry. Blue (DAPI) staining identifies cell nuclei. β-actin was the internal control.

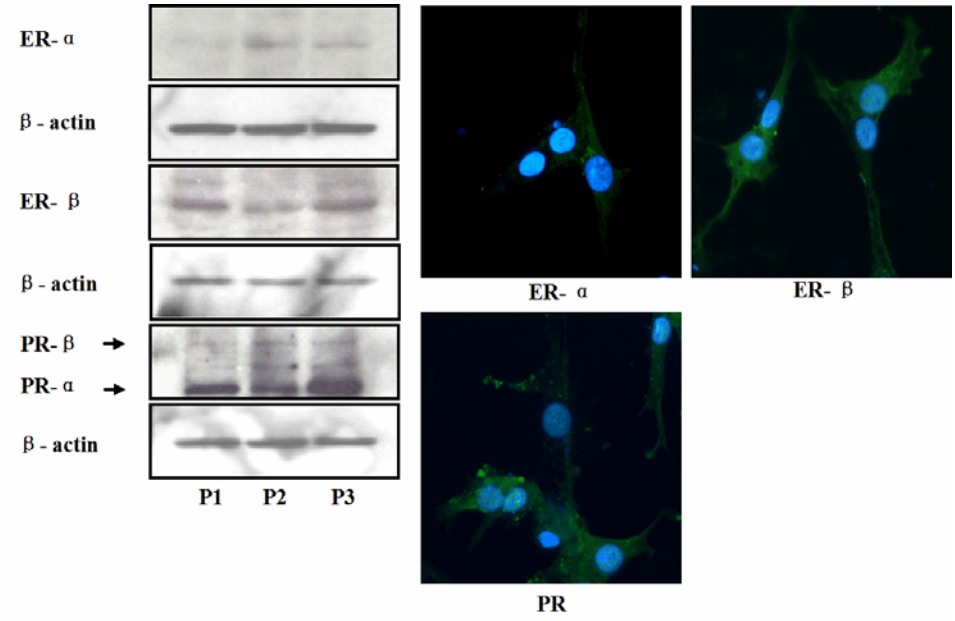

Figure 4. The expression patterns of steroid hormone receptors in primary cultured human granulosa cells. After preculture for 24h, proteins were isolated from primary granulosa cell cultures of three patients (designated by P1, 2, and 3). The expressions of steroid hormone receptors (ER-α,β and PR-α,β) were assessed by western blotting and immunocytochemistry. Blue (DAPI) staining identifies cell nuclei. β-actin was the internal control.

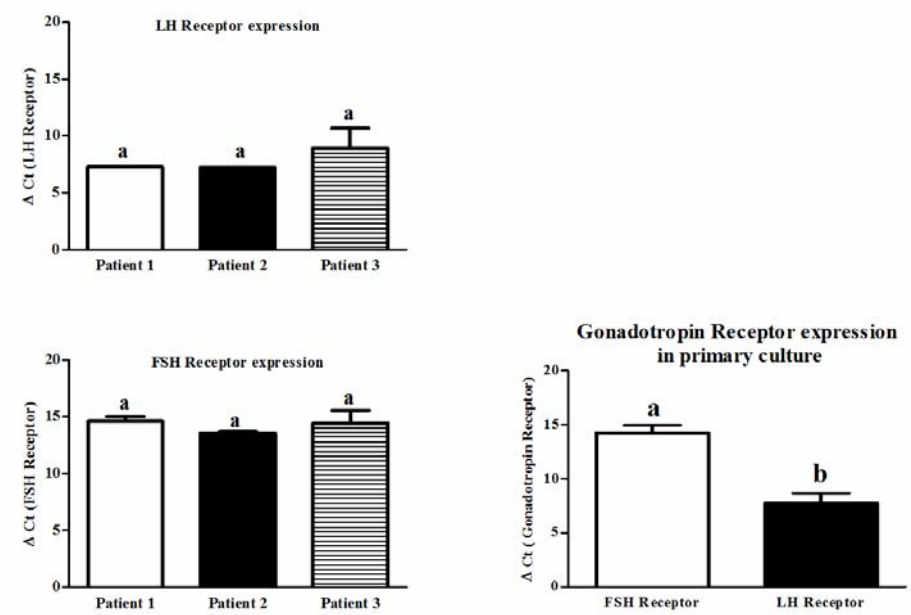

Figure 5. The mRNA expression of LH and FSH receptor in primary cultured human granulosa cells. The cell culture condition was same as described in Fig. 1. The mRNA was isolated from primary granulosa cell cultures of three patients (designated by P1, 2, and 3). The mRNA levels of FSH and LH receptor were assessed by real-time PCR. The expression level of LH receptors was higher than that of FSH receptors. Ct (Threshold cycle) levels are inversely proportional to the amount of target mRNA in the sample, ie, the lower the Ct level, the greater the amount of target mRNA in the sample. Results from three independent experiments were presented as the mean ± SD. Means without a *common letter* are significantly different ($P < 0.05$).

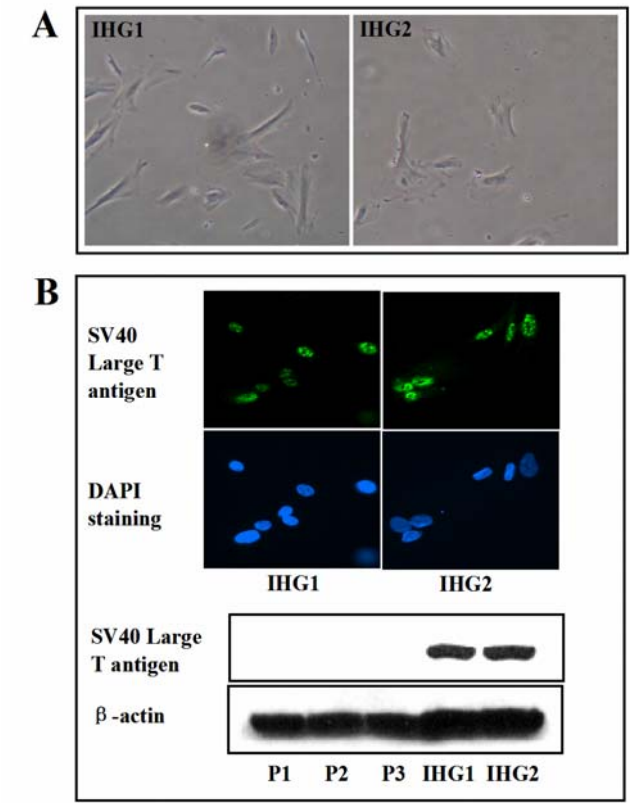

Figure 6. Cell morphology, immunofluorescence and western blot analysis in SV40 large T antigen transfected granulosa cells. (A) Cell morphology. After two weeks of transfection by SV40 large T antigen using FuGENE 6 transfection reagent, the transfected granulosa cells (designated IHG1 and IHG2) exhibited elongated and spindle-shape. (B) The presence of SV40 large T antigen. The SV40 large T antigen was stained on immunofluoresence in the nuclei (stained with DAPI as an internal control) of transfected cells (designated IHG1 and IHG2). The SV40 large T antigen was also detected in two transfected cells (designated IHG1 and IHG2) but not in primary granulosa cell cultures of three patients (designated by P1, 2, and 3) by western blotting. β-actin was internal control.

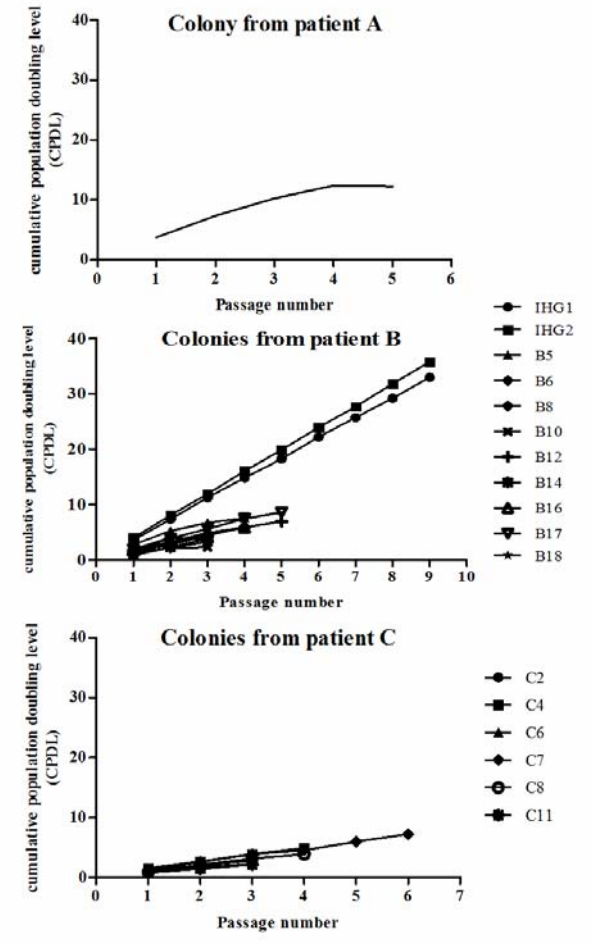

Figure 7. The growth potential of transfected human granulosa cell colonies derived from three different patients. Extended lifespan were evaluated by serial sub-cultures and direct cell counting. In each subculture, cells were plated at 2 x 10^5 seeding densities and cultured for 5 days. Initially, 18 clones were derived from three different patients. 16 clones were not able to bypass senescence before confluence but two clones (designated IHG1 and 2) derived from patient B were able to bypass senescence and acquire an extended lifespan (grew more than 30 CPDL). CPDL (Cumulative Population Doubling Level).

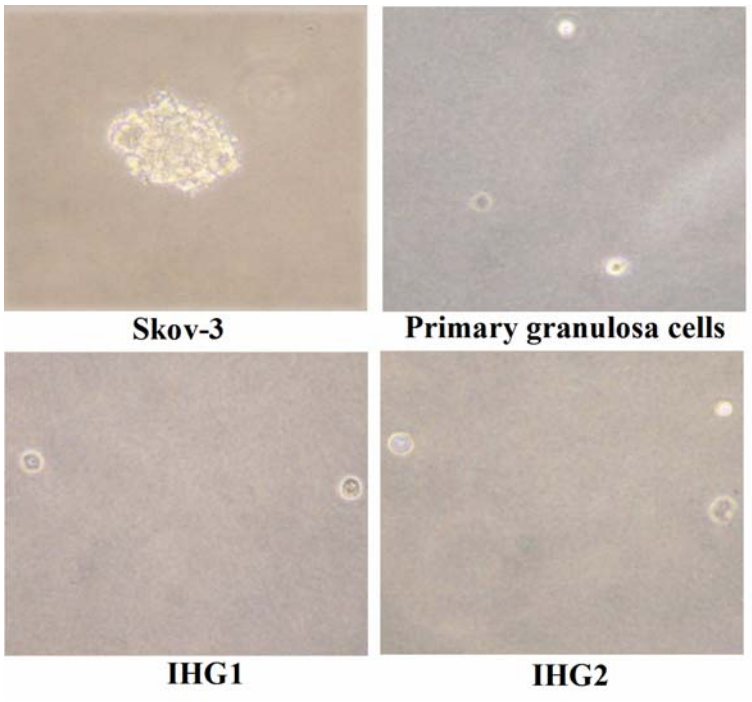

Figure 8. The anchorage-dependent growth of transfected granulosa cells. Anchorage-independent growth (AIG) capability was determined by assessing the colony-forming efficiency of cells suspended in soft agar. After preculture for 24h, granulosa cells (1×10^4) suspended in culture medium containing 0.33% "soft" agarose were overlaid on top of 0.5% "hard" agarose layer. Additional culture medium was added on the top and cultured for 4-weeks. SKOV-3 formed large colonies in soft-agar but primary cultured and transfected granulosa cells (designated IHG1 and IHG2) failed to form colony. Ovarian carcinoma cell line (SKOV-3) and primary cultured granulosa cells were used as the positive and negative controls respectively.

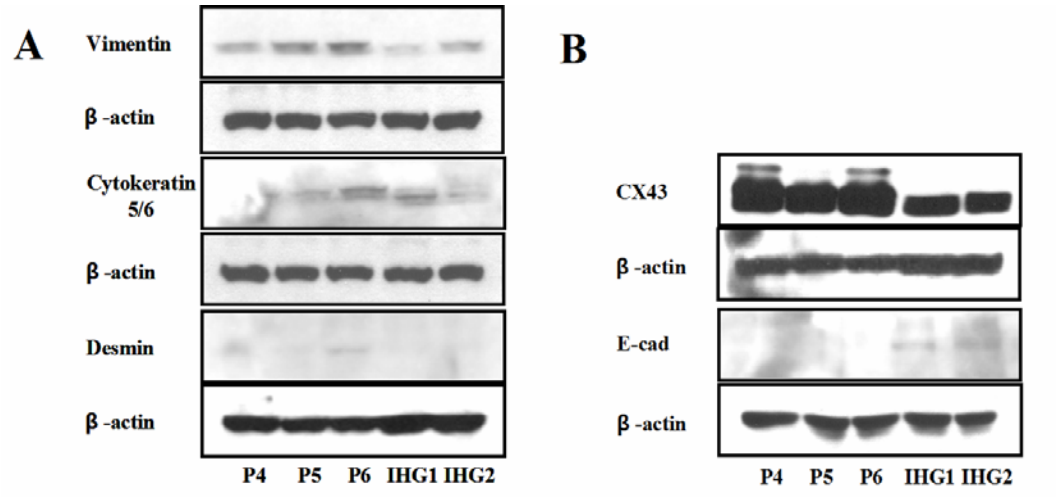

Figure 9. The expression patterns of cytoskeletal proteins and adhesion molecules in immortalized human granulosa cell lines. The cell culture condition was same as described in Fig. 1. The expressions of (A) cytoskeletal proteins (vimentin, cytokeratin 5/6 and desmin) and (B) adhesion molecules (Cx43 and E-cadherin) in immortalized granulosa cells (designated IHG1 and IHG2) were assessed by western blotting and compared with the results of primary cultured granulosa cells from three patients (designated P4, 5, 6).

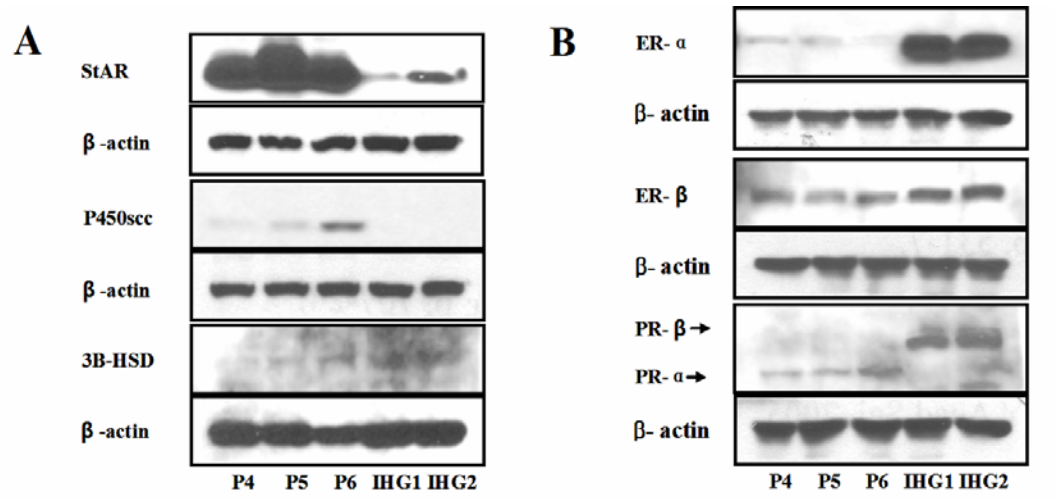

Figure 10. The expression patterns of steroidogenic enzymes and steroid hormone receptor in immortalized human granulosa cells. The cell culture condition was same as described in Fig. 1. The expressions of (A) steroidogenic enzymes (StAR, P450scc and 3β–HSD) and (B) steroid hormone receptors (ER-α,β and PR-α,β) in immortalized granulosa cells (designated IHG1 and IHG2) were assessed by western blotting and compared with the results of primary cultured granulosa cells from three patients (designated P4, 5, 6).

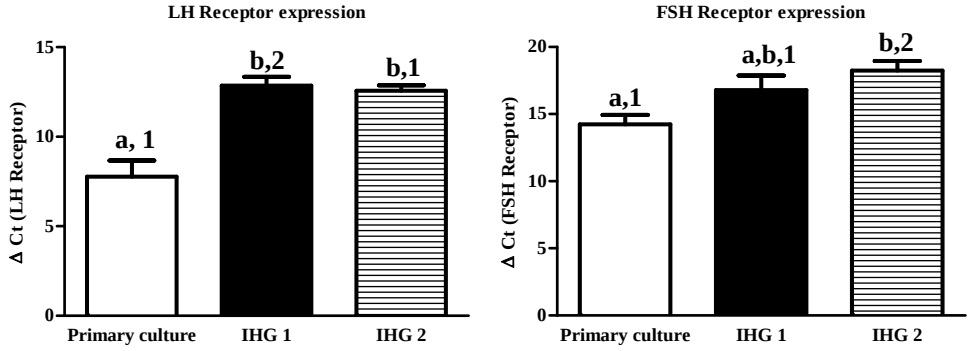

Figure 11. The expression patterns of gonadotropins receptors in immortalized human granulosa cells. The cell culture condition was same as described in Fig. 1. The mRNA levels of FSH receptor and LH receptor in both primary granulosa cell cultures of three patients and immortalized granulosa cell lines (designated IHG 1 and 2) were assessed by real-time PCR. Ct (Threshold cycle) levels are inversely proportional to the amount of target mRNA in the sample. Means without a *common letter* (a,b: One-way ANOVA and 1,2: Non-parametric Kruskal–Wallis test) are significantly different ($P < 0.05$).

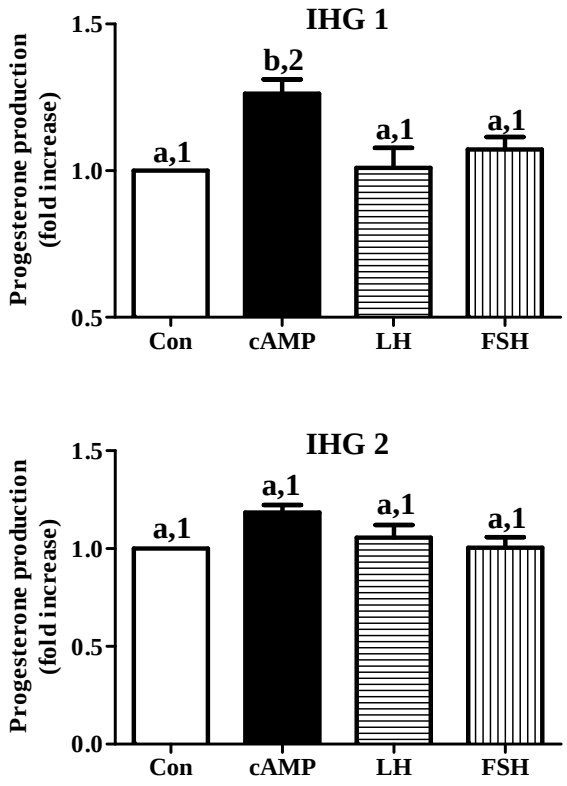

Figure 12. The progesterone production in two immortalized human granulosa cell lines. IHG 1 and 2 cell lines were cultured with and without 8-Br-cAMP (1mM), FSH (50 ng/ml) or LH (50 ng/ml) for 24h. Progesterone (P4) concentration was determined by Progesterone Correlate-EIA™ kits according to the manufacturer's instructions. Increased progesterone production was obtained after 24h incubation in presence of 8-Br-cAMP in IHG1 but little change in IHG2. However, no difference of progesterone production was found after treatment of LH or FSH in both cell lines Results from three independent experiments were presented as the mean ± SD. Means without a *common letter* (a,b: One-way ANOVA and 1,2: Non-parametric Kruskal–Wallis test) are significantly different ($P < 0.05$).

4. Gonadotropin-releasing hormone -I and II induce apoptosis in human granulosa cells

4.1. Introduction

Apoptosis is the biological process by which unwanted cells are eliminated in response to the developmental signals or toxic stimuli. The major features of apoptosis are cell shrinkage, DNA fragmentation, cell membrane blebbing and apoptotic body formation (279, 280). Apoptosis plays an important role in the processes that maintain the reproductive apparatus (281, 282). During development and growth of human ovarian follicles, only a limited number of follicles proceed to the ovulatory stage, whereas more than 99% of follicles undergo the apoptotic process of atresia (1). Recent studies have revealed that apoptosis of ovarian granulosa cells plays an important role in follicular atresia (79, 283). Granulosa cells maintain the developing oocyte until its release from the follicle during ovulation and also synthesize hormones that are related to oocyte maturation and ovulation. Many investigators have studied factors that directly or indirectly regulate apoptosis of granulosa cells. Recent studies have suggested a physiologic role of gonadotropin-releasing hormone (GnRH) in granulosa cell apoptosis (284).

GnRH is the important hypothalamic regulator of normal human reproductive functions (223, 224). GnRH is produced and released by hypothalamic secretory neurones and carried to the anterior pituitary cells through the portal vascular system. In the anterior pituitary, GnRH induces the production and the release of the gonadotropins (LH and FSH). The effects of GnRH are mediated by a cell surface receptor (GnRH-R) belonging to the G protein-coupled receptor superfamily (285, 286). In addition to its well-known roles in regulating the hypothalamus-pituitary-gonadal axis, GnRH has been found in extra-hypothalamic regions of the central nervous system (86). The expression of GnRH-I and II has also been identified in human luteal-granulosa cells (220). GnRH-I and GnRH-I receptors have been localized in human reproductive tissues, such as the placenta (226), the ovary (287), the endometrium (99), and testis (227). Functional studies have established that GnRH-I regulates steroidogenesis and inhibits cell growth in human ovarian cells (223). GnRH-I mRNA in granulosa cells of primary, preantral and antral follicles has been identified from in situ hybridization studies (287). Recent studies from our laboratory have also confirmed GnRH-II expression in human granulosa-luteal cells from IVF patients, immortalized ovarian surface epithelial (OSE) cells and many ovarian cancer cell types (287). GnRH-II is also broadly distributed and highly expressed throughout the extra-

hypothalamic regions, including ovarian (288), endometrial (102), and breast tissue (289). Only the truncated but not the functional forms of GnRH-II receptors are known to exist in humans even though GnRH-II receptor mRNA expression has been established in multiple tissues, such as placenta, breast, endometrium, and ovary (102, 289). Hence, the functions of GnRH-II in reproductive tissues and the mechanisms involved remain unclear.

The direct effects of GnRH-I on apoptosis in granulosa cells have been investigated by several researchers, GnRH-I increases the incidence of apoptosis in porcine granulosa cells (78, 290) and down-regulates the proliferation of human granulosa cells (188, 291). Because of their close association with the oocyte, increased apoptosis of granulosa cells may influence the development and quality of the oocyte (292, 293), increase the occurrence of empty follicles, poor oocyte fertilization, and result in lower pregnancy rates (156). However, the direct effects of GnRH-I and GnRH-II on apoptosis in human granulosa cells and the underlying mechanisms involved remain unknown.

The purpose of this study was to characterize the effects of GnRH-I and II on granulosa cells, using human immortalized granulosa cells as the experimental model. We used the TUNEL (terminal deoxynucleotidyl-transferase (TdT)-mediated biotin-

dUTP nick-end labeling) assay to investigate apoptosis induced by GnRH-I and GnRH-II. Further, since FSH is a major survival factor of ovarian follicles, we examined its anti-apoptotic effects on human granulosa cells following co-treatment with GnRH-I or II.

4.2. Materials and methods

4.2.1. Experimental design

In each set of experiments, human immortalized granulosa cells were cultured without or with GnRH-I or GnRH-II, each at a dose of 10^{-7} mol/L. In separate experiments involving the same three groups, each of following treatments was added to the culture medium of the GnRH-I and GnRH-II groups (but not to that of controls) at the specified time: 1) 10^{-7} mol/L of the GnRH antagonist, antide, was added 2 hours before GnRH-I or GnRH-II; and 2) 50 ng/ml FSH was added to the culture medium at the same time as GnRH-I or GnRH-II treatment. All experiments were cultured in duplicate or triplicate and evaluated in a time-dependent manner. TUNEL assays, immunoblotting and expression levels of caspases were used to compare the effects of these various treatments alone or in combinations on apoptosis.

4.2.2. Reagents and antibodies

GnRH I (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2), GnRH II (pGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH_2), and the GnRH antagonist, antide (*N*-Ac-D-Nal1-D-Cpa2-D-Pal3-Ser4-Lys(Nic)5-D-Lys(Nic)6-Leu7-Ilys8-Pro9-D-Ala10-NH_2), were purchased from Bachem (King of Prussia, PA). All stock solutions were aliquoted, stored at -20°C, and diluted in cell culture medium immediately prior to use.

4.2.3. Cell culture

Human granulosa cells were obtained from IVF procedures and immortalized with SV40 large T antigen as described previously (163). The human immortalized granulosa cells were cultured in M199/MCDB105 (1:1) supplemented with 10% FBS (Hyclone) at 37°C in a humidified atmosphere of 5% CO_2 in air. After culturing for 2 days, the cell culture dishes were washed one time with culture medium and serum starved for 6 hours before specific treatment.

4.2.4. Immunoblotting analysis

After each treatment, cells were washed two times with PBS and lyzed in RIPA buffer containing phosphatase inhibitor cocktail I (Sigma Chemical, St. Louis,

MO). The protein concentration of the supernatants was determined by the Bio-Red DC protein assay (Bio-Rad Laboratories, Inc.). Samples fractionated on SDS-PAGE at constant voltage were then transferred onto nitrocellulose membranes for 2h. The membrane was blocked by incubation in 10ml PBS containing 5% skim-milk powder at room temperature for 1h. The membrane was washed in PBS containing 0.05% Tween 20 (Tween-PBS; Sigma Chemical, St. Louis, MO), and then incubated overnight with the specific primary antibodies at 4°C. Secondary antibodies diluted in the same solution were then added to each membrane, incubated for 1 h at 37°C, rinsed three times with PBS containing 0.05% Tween 20. Each band on western blotting was quantitated with the Scion Image Software (Scion, Frederick, MD) using B-actin as the internal control.

4.2.5. TUNEL assay

DNA strand breaks in apoptotic cells were measured by the TUNEL assay using the In-situ Detection Kit, POD (Roche Molecular Biochemicals, Germany). Samples were fixed with 4% paraformaldehyde in PBS for 15 min and incubated in a 0.1% ice-cold Triton X-100 solution for permeabilization according to the manufacturer's instructions. Cell were then wished 3 times with PBS and reacted with

50 µl of the TUNEL reaction mixture at 37°C for 60 min in a dark, humidified chamber. Cells were washed three times in PBS and incubated for 30 minutes with 50 µl of the Converter-POD (Roche Diagnostics, Castle Hill, Australia) followed by another 15 minutes with DAB to ensure the detection of TUNEL labeled cells. Under light microscopy, the number of TUNEL-positive cells per high-power field (100x) was counted and expressed as a percentage of the total number of cells present in that field.

4.2.6. Statistical analysis

Results from three independent experiments were presented as the mean ± SD. Data were first assessed by two-way analysis of variance (ANOVA) with time as the repeated measure. Significant results were followed by one-way factorial and repeated measures ANOVA for between- and within-group differences respectively and corresponding significant results were further assessed by Tukey's multiple comparison tests. Statistical significance was defined at a P level of <0.05. Since normality of data was not assumed, results were furthered analyzed by the non-parametric equivalents of Kruskal-Wallis test and Friedman test for between- and

within-group comparison respectively. Because the pattern of statistical significance remained the same, results were reported according to ANOVA.

4.3. Results

4.3.1. Apoptotic effects of GnRH-I and II in human granulosa cells

To investigate that GnRH-I and II induced apoptosis, we performed TUNEL assays on cells obtained from cultures at 24, 48 and 72 hours to identify apoptotic cells, characterized by the inclusion of densely stained circular bodies representing fragmented DNAs of apoptosis. GnRH-I or II treatment significantly increased the number of these apoptotic or TUNEL-positive cells in a time-dependent manner compared to controls. These apoptotic changes induced by GnRH-I and II were clearly observed by 48 hours and reached and peaked at 72 hours. The number and distribution of TUNEL-positive cells following GnRH-I or II treatment were similar (Fig. 13).

To further confirm that GnRH-I and II induced apoptosis, Western blot analysis was used to evaluate the expression levels of some of the critical intracellular mediators of apoptosis, the initiator caspase-8 and the effectors caspases-3 and 7 in human immortalized granulosa cells treated with GnRH-I or II (10^{-7} mol/L) in a time-

dependent manner (24, 48, 72hr). As shown in Fig. 14, both GnRH-I and II significantly activated caspase-8, 3 and 7. Furthermore, these data from Western blot analysis were consistent with our TUNEL assay results. In the intracellular proteolytic cascade of apoptosis, caspase-8 directly cleaves the relevant procaspases and activates effectors such as caspases-3 and 7 downstream (183). Hence, our results revealed that GnRH-I and II indeed induced apoptosis and triggered the corresponding cascade mediated by caspases.

4.3.2. GnRH antagonist, antide, inhibited the ability of GnRH-I and II mediated apoptosis

In contrast, antide, when added at a dose of 10^{-7} mol/L to the media prior to GnRH-I and II treatment, effectively blocked the TUNEL-positive changes observed with GnRH-I or II alone (Fig. 15A) in human granulosa cells. Further, the corresponding expression levels of caspase-3 (a key effector caspase) as assayed by Western blotting were significantly attenuated (Fig. 15B). Because the functional receptor for GnRH-II has not yet been identified in human, and antide, a GnRH-I antagonist, successfully blocked GnRH-II induced apoptotic changes in our granulosa

cell cultures, our findings strongly suggest that both GnRH-I and II mediate apoptosis by binding to GnRH-I receptors.

4.3.3. FSH protected human granulosa cells from undergoing GnRH-I and II mediated apoptosis

FSH is a major survival factor for early antral follicles (233). In vitro, FSH prevents atresia of cultured preovulatory follicles (227). To investigate these anti-apoptotic effects of FSH, human immortalized granulosa cells were cultured with both FSH (50ng/ml) and GnRH-I or II (10^{-7} mol/L). The time-dependent increase in TUNEL-positive cells induced by GnRH-I or II were effectively attenuated by FSH (50ng/ml) co-treatment as shown in Fig. 16A. Correspondingly, increased expression of cleaved caspases 8, 3 and 7 on western blot analysis induced by GnRH-I or II from 48 to 72 hours were attenuated by FSH co-treatment (Fig. 16B). These results demonstrate that FSH indeed acts as a survival factor, protects human granulosa cells from undergoing apoptosis and attenuates the apoptotic effects of GnRH-I and II.

4.4. Discussion

Granulosa cells play an important role in regulating ovarian physiology, maintaining and controlling ovarian function, growth and differentiation (294, 295). The growing oocyte is surrounded by layers of granulosa cells. Granulosa cells synthesize and secrete a variety of growth factors and cytokines that may regulate the differentiation and growth of the oocytes. Hence, communication between the granulosa cells of the growing follicle and the oocyte is essential for its development and maturation (296). Further, apoptosis of granulosa cells is not only related to follicle atresia, but may be linked to poor oocyte quality and lower pregnancy rates following suboptimal ovarian stimulation with FSH and GnRH agonist exposure during in vitro fertilization treatment for infertility.

Our study has focused on the roles of GnRH-I and II on apoptosis in human granulosa cells, the underlying molecular and cellular processes involved, and the anti-apoptotic effects of FSH. Our results show that GnRH-I and II can indeed increase apoptosis in human granulosa cells in a time-dependent manner, findings that are consistent with the apoptotic effects of GnRH agonists documented in previous studies (284, 297). We have also demonstrated that GnRH-I and II induce apoptosis through

the GnRH-I receptor since antide, a GnRH-I antagonist, can successfully block apoptosis induced by GnRH-II in human immortalized granulosa cells.

Apoptosis is a physiological process by which unwanted cells are eliminated in response to the developmental signals or toxic stimuli without inducing an immune response or inflammatory reaction (279, 280, 298). Apoptosis is mediated by the activation of a family of cysteine proteases, known as caspases. Among the caspases, caspase-3 and 7 are effectors responsible for cleaving a variety of key proteins in the cell, including poly (ADP-ribose) polymerase (PARP) and lamins. Caspase-8 is an initiator which activates the effector caspases, producing an amplifying chain reaction. Our results show that both GnRH-I and II can significantly activate initiator caspase-8 and effector caspases-3 and 7 (Fig. 14).

FSH is the most important survival factor for follicle growth in the preovulatory phase, and a lack of FSH at the critical time will lead to follicular atresia through apoptosis (188, 281, 299) . It has been well documented that FSH increases estradiol and progesterone production by granulosa cells in vitro. Estrogens, in association with gonadotropins, are known to effect granulosa cell growth (300). Furthermore, recent evidence has shown that progesterone acts as a survival factor which prevents apoptosis in rat granulosa cells by inhibiting the cell oxidation pathway

(174). FSH is widely treated during in vitro fertilization (IVF) procedure to stimulate the development of multiple follicles after pituitary down-regulation with GnRH agonist. Hence, these two hormones can regulate apoptosis of granulosa cells but in opposite directions - GnRH agonist acts as an inducer while FSH acts as an inhibitor of apoptosis (78, 79, 188). We therefore hypothesized that FSH could similarly counteract apoptosis induced by GnRH-I or II in human granulosa cells. Indeed, GnRH-I or GnRH-II induced TUNEL-positive reactions and cleavage of caspases 8, 3 and 7 were attenuated by FSH co-treatment. These data confirms that FSH protects granulosa cells from undergoing apoptosis induced by GnRH-I or GnRH-II.

We previously characterized, in detail, mRNA expression of GnRH-I, GnRH-II, and GnRH-I receptors not only in our immortalized cell lines, but also in human luteinized granulosa cells obtained from IVF procedures (301). In the same study, we have further localized by immunocytochemistry, the presence of GnRH-I, GnRH-II, and GnRH-I receptors in the granulosa cell layer of preovulatory follicles as well as granulosa luteal cells of the corpus luteum from human ovaries. Hence, the findings from our current study further attest to our original suggestion that immortalized granulosa cell lines, in addition to primary cultured granulosa cells, provide a suitable experimental model for functional studies on the autocrine effects of GnRH-I and

GnRH-II and their receptors (301) in the human ovary.

Our *in vitro* findings appear to be incongruent with clinical practice when the commonest IVF regimen involves GnRH agonist treatment before and during FSH administration as a strategy to increase multiple follicle development and growth, hence, oocyte yield. However, this apparent discrepancy can be explained. First, our findings are not referring to the known endocrine effects of continuous GnRH or GnRH agonist treatment on the pituitary-ovarian axis. Rather, we are modeling the effects of GnRH-I and GnRH-II produced locally in the ovary on granulosa cell function. Second, if our *in vitro* findings were to apply to the clinical setting, the availability of FSH from exogenous administration would counteract the apoptotic effects of the GnRH system, just as in our *in vitro* model. Our *in vitro* findings may also have clinical relevance in women at risk of developing ovarian hyperstimulation syndrome, when FSH administration is withdrawn ("coasting") to allow high estradiol levels to fall to a lower range before hCG administration while GnRH agonist treatment is continued. Indeed, low pregnancy rates have been reported if coasting is greater than 4 days (302). It is tempting to speculate that, among other causes, the GnRH system may induce apoptosis of the granulosa cells after ambient FSH levels have declined to a critical threshold during prolonged coasting, which in turn, affect

oocyte quality and consequently, pregnancy rates. Finally, with the newer ovarian stimulation regimen using GnRH antagonists instead of GnRH agonists to prevent premature LH surge, future studies evaluating the role of GnRH antagonists alone on granulosa cell apoptosis and the related caspase signaling pathways will be of interest.

In conclusion, our study shows that in addition to its well-established actions on the pituitary–gonadal axis, GnRH-I (as well as GnRH-II) can directly induce granulosa cell apoptosis in human. This raises a potentially important role of GnRH-I or -II in the regulation of atresia and follicle development. The direct apoptotic effects of GnRH-I and II are mediated by GnRH-I receptors. The intracellular signals of apoptosis induced by GnRH-I or II, in turn, are mediated by activation of the proteolyic caspase cascade, involving caspase-8 (the initiator) and caspase-3 and caspase-7 (the effectors). In addition, FSH protects human granulosa cells from undergoing apoptosis induced by GnRH-I or II. Our study provides a novel insight into the caspase signaling pathways involved in GnRH-I or II induced human granulosa cell apoptosis. Moreover, our in vitro model systems suggest potential mechanisms responsible for follicular atresia through regulation of apoptosis of granulosa cells by GnRH-I or II, which warrants further investigation.

4.5. Figures

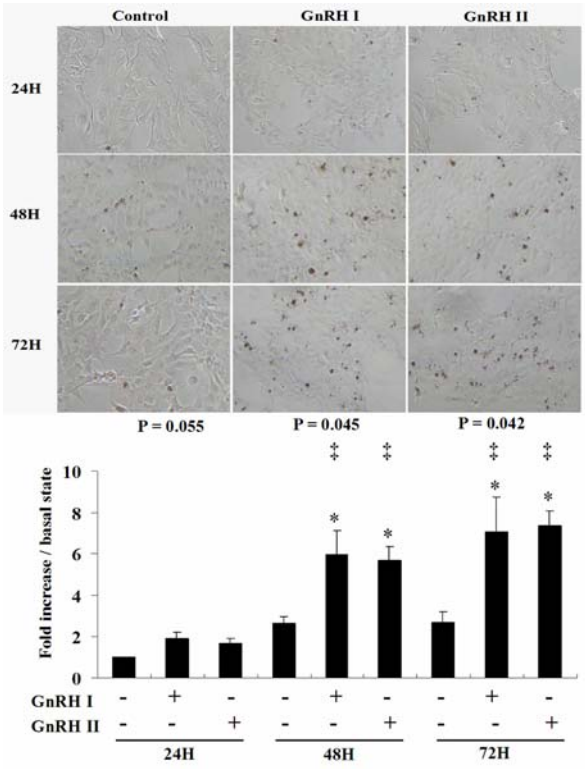

Figure 13. Time-dependent apoptotic effects of GnRH-I and II on human immortalized granulosa cells. Apoptotic nuclei were identified with the TUNEL assay after treatment with GnRH-I or II (each at a dose of 10^{-7} mol/L). GnRH-I and II significantly increased the number of TUNEL-positive cells after 48hr in a time-dependent manner. The number and distribution of apoptotic or TUNEL-positive cells following GnRH-I or II treatment were similar. Significant within-group differences on ANOVA were followed by pairwise comparison (Tukey's test) with (*) and (†) denoting significant differences from values at 24h and 48h respectively. Significant between-group differences on ANOVA at each time were similarly compared. The p value represents between-group differences at each time point with (‡) denoting significant differences from control values.

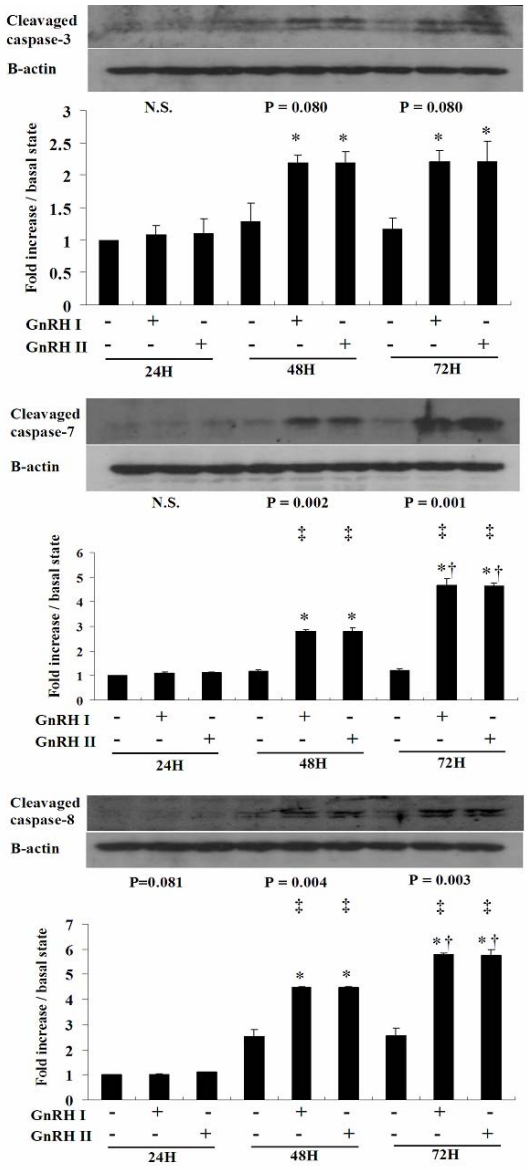

Figure 14. GnRH-I and II induced cleavage activities of caspase 3, 7 and 8 on Western blot analysis. Human immortalized granulosa cells were treated with GnRH-I or II (each at a dose of 10^{-7} mol/L) in a time-dependent manner (24, 48, 72hr). Both GnRH-I and II significantly activated the initiator caspases-8 and the effector caspases-3 and 7. See Figure 13 for annotations.

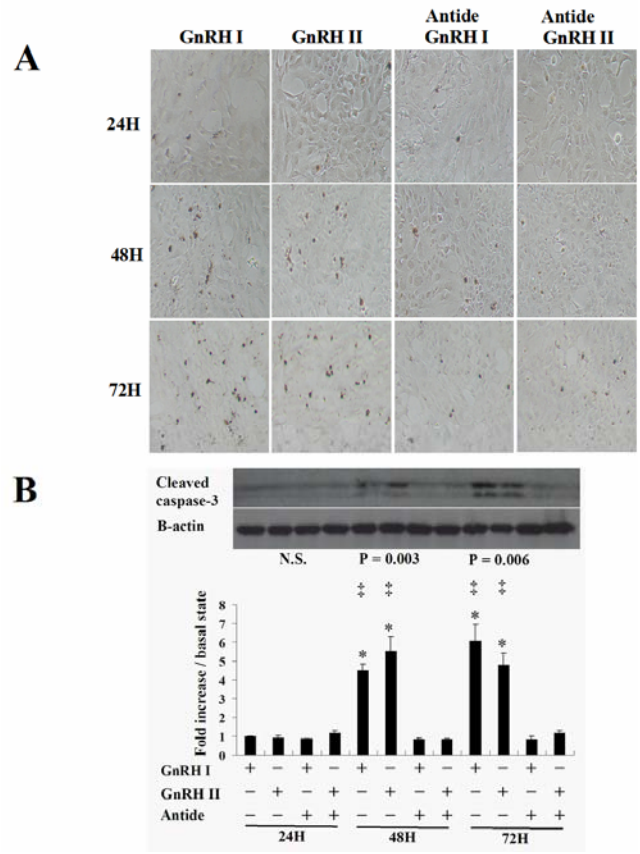

Figure 15. GnRH antagonist, antide, attenuated the ability of GnRH-I and II mediated apoptosis. (A) In the presence of antide (10^{-7} mol/L), the apoptotic effects of GnRH-I or II on human immortalized granulosa cells were significantly attenuated, reducing the number of apoptotic or TUNEL-positive cells seen. **(B)** Western blot analysis showed that the corresponding expression levels of caspase-3 (a key effector of apoptosis) induced by GnRH-I or II were effectively blocked by antide. See Figure 13 for annotations except (‡) now denotes significant differences from values in treatment groups with antide added.

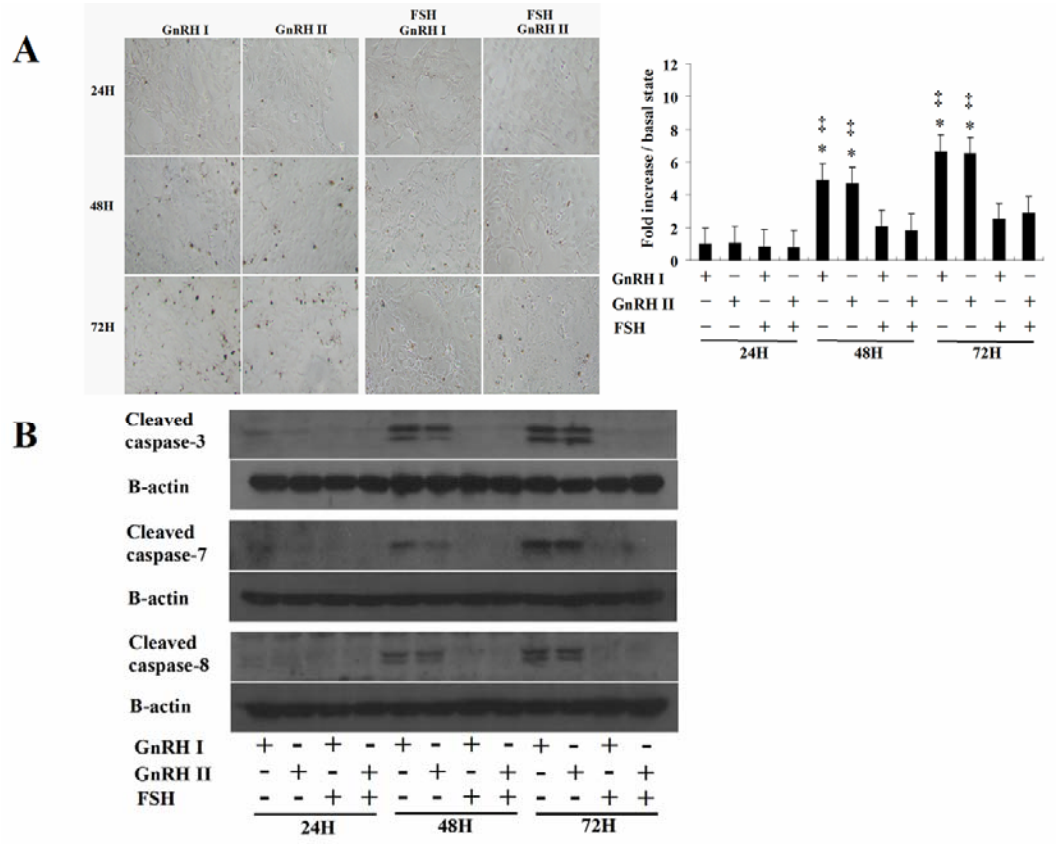

Figure 16. FSH as a survival factor, preventing apoptosis of human immortalized granulosa cells induced by GnRH-I or II. (A) Human immortalized granulosa cells were treated with FSH (50ng/ml) and GnRH-I or II (10^{-7} mol/L). GnRH-I and II significantly increased the number of TUNEL-positive cells after 48 hours in culture. The number of these apoptotic cells was markedly reduced with FSH cotreatment. **(B)** GnRH-I or II induced cleavages of the initiator, caspase-8, and the effectors, caspase-3 and 7, were blocked by FSH (50 ng/ml) cotreatment.

5. Gonadotropin-releasing hormone -I or II interacts with IGF-I/akt but not connexin 43 in human granulosa cell apoptosis

5.1. Introduction

Gonadotropin-releasing hormone (GnRH) is the central hypothalamic regulator of reproductive function (223). In the anterior pituitary, GnRH stimulates the synthesis and the release of the gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH). Analogs of GnRH are widely used in assisted reproduction technology (ART) as part of the ovarian stimulation protocols to prevent a premature endogenous LH surge (303). In humans, GnRH and its receptors have been found in extra-pituitary tissues such as the ovary (304), breast (305), and placenta (306). The expression of GnRH receptors has also been demonstrated in human ovarian granulosa cells (228).

In addition to producing important steroid hormones and growth factors, granulosa cells play a significant role in oocyte maturation until its release from the follicle (295). The health of granulosa cells can influence oocyte quality and hence, future embryo development. GnRH has been shown to have anti-proliferative effects on granulosa cells from rats (230), pigs (231), and humans (83) but the underlying

mechanisms involved are unclear. Potential mechanisms include: cell signaling through the extrisnisc and/or intrinsic pathways of apoptosis, interference of mitogenic activities of growth factors such as insulin-like growth factor-I (IGF-I), and/or disruption of gap junctions for effective cell-to-cell communications.

Apoptosis can occur through either the extrinsic or the intrinsic pathways in the cell. The extrinsic pathway requires the activation of the intracellular caspase cascade. In contrast, the intrinsic pathway is initiated through the Bcl-2 family proteins. Bcl-2 family can be divided into two sub-families: anti-apoptotic Bcl-2 proteins such as Bcl-X(L), Bcl-w, Mcl-1, A1, Bcl-Rambo, and Bcl-G, and pro-apoptotic proteins such as Bax, Bak, and Bok (307). Apoptosis through the intrinsic pathway involves interactions between these anti- and pro-apoptotic members of the Bcl-2 family (308).

We have recently demonstrated that GnRH-I or II can induce apoptosis in immortalized human granulosa cells by activating the caspase signaling cascade (208) of the extrinsic pathway (309). However, some Bcl-2 protein family members are also involved in granulosa cell apoptosis - Bad, Bax, and Bcl-X(L) in mice (310), Bax, Bcl-2 and Bcl-X(L) in rats (179), and Bax and Bcl-X(L) in primates (178). Bax and Bcl-X(L) have also been shown by immunostaining to localize to human luteinized granulosa cells from atretic follicles obtained from women undergoing IVF treatment

(178) and Bcl-2 in the corpus luteum from ovaries in women undergoing surgery. Moreover, the anti-proliferative effects of GnRH agonists can also activate Bcl-2 family of the intrinsic pathway in rat granulosa cells (311). Whether GnRH-I or II can also activate some of the Bcl-2 family members of the intrinsic pathway in human granulosa cells is unknown.

IGF-I is known to have anti-apoptotic actions in human tissues such as the ovary (1) and breast (312). IGF-I can stimulate human granulosa cell proliferation (237) and increase steroid secretion and responsiveness to gonadotropins in ovarian follicles of sheep (33) and humans (240). Recent studies also suggest that GnRH agonists can interfere with IGF-I receptor expression and activation in androgen-independent human prostate cancer cells (241) and mouse pituitary cells (242). An important IGF-I receptor signaling mediator is Akt. IGF-I induced proliferation is mediated by Akt phosphorylation in human granulosa cells obtained from women undergoing IVF (313). However, there is currently no information on whether GnRH–I or II can affect the proliferative actions of IGF-I in human granulosa cells.

Gap junctions mediate cell homeostasis by regulating the passage of signaling molecules smaller than 1000 dalton across the cell membrane. Each channel of gap junction is composed of the connexin (Cx) family. Cx43 is a major gap junction

protein in granulosa cells of many species including humans (258, 314) and plays important roles in human granulosa cell development and apoptosis (315). GnRH analogs can induce phosphorylation of Cx43 and reduce Cx43 mRNA expression in rat granulosa cells (246). However, the effects of GnRH-I or II on gap junctions and Cx43 in human granulosa cells are unknown.

We therefore conducted the following studies, using primary cultured or immortalized human granulosa cells as the study model, to evaluate the effects of GnRH-I or II on 1) the expression and phosphorylation of the Bcl-2 family members Bcl-2, Bad, Bim and Bax; 2) the mitogenic actions of IGF-I system (IGF-I receptor expression/phosphorylation and Akt signaling); and 3) gap junction (Cx43 expression and phosphorylation).

5.2. Materials and methods

5.2.1. Cell culture

As our original SVOG granulosa cell line (163) was no longer available, we immortalized two new human granulosa cell lines using FuGENE 6 transfection reagent (Roche Molecular Biochemicals, Germany) according to the manufacturer's instructions and characterized them in greater detail than the SVOG cell line (163) used in our previous studies (208). Granulosa cells were also collected from IVF

patients for selective experiments for comparison. The cell suspension was gently spin in a centrifuge at 500×*g* for 10min, and the supernatant removed. The final pellet was resuspended in 5 ml of M-199/MCDB 105 (1:1 mixture) with 10% FBS. Granulosa cells can be separated from other cell types by centrifuging for 10 min at 2800*g* on a single density Percoll layer. Isolated granulosa cells were then cultured in M-199/MCDB 105 (1:1 mixture) with 10% FBS and maintained at 37°C in humidified atmosphere of 5% CO_2 in air. After culturing for 1 day, the cells were washed one time with medium before GnRH-I, II, and/or IGF-I treatment.

5.2.2. Reagents and antibodies

GnRH I (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2), GnRH II (pGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH_2), and the GnRH antagonist, antide (*N*-Ac-D-Nal1-D-Cpa2-D-Pal3-Ser4-Lys(Nic)5-D-Lys(Nic)6-Leu7-Ilys8-Pro9-D-Ala10-NH_2), were purchased from Bachem (King of Prussia, PA). Anti-phospho-Cx43, anti-Cx43, anti-capapse-3, anti-phospho-IGF-IR, anti-ERK1/2, anti-phospho-ERK1/2, anti-phospho-Bad, anti-phospho-Bcl-2, ant-Bok, anti-Bax, anti-Bim, anti-IGF-IR, anti-total-Akt and anti-phospho-Akt antibody were purchased from Cell Signaling

Technology (Beverly, MA, USA). Anti-GnRH-receptor antibody *was* purchased from Neomarkers (Fremont, CA, USA).

5.2.3. Western blot analysis

After each treatment, cells were washed two times with PBS and lyzed in RIPA buffer containing phosphatase inhibitor cocktail I (Sigma Chemical, St. Louis, MO). The protein content of the supernatants was determined by the Bio-Red DC protein assay (Bio-Rad Laboratories, Inc.). Samples fractionated on SDS-PAGE at constant voltage were then transferred onto nitrocellulose membranes for 2h. The membrane was blocked by incubation in 10ml PBS containing 5% skim-milk powder at room temperature for 1h. The membrane was washed in PBS containing 0.05% Tween 20 (Tween-PBS; Sigma Chemical, St. Louis, MO), and then incubated overnight with the specific primary antibodies at 4°C. Secondary antibodies diluted in the same solution were then added to each membrane, incubated for 1 h at 37°C, rinsed three times with PBS containing 0.05% Tween 20. Each band on western blotting was quantitated with the Scion Image Software (Scion, Frederick, MD) using B-actin as the internal control.

5.2.4. Cell proliferation and viability

Cell proliferation and viability were evaluated by culturing cells for 1, 2 and 3 days. After these periods, cells were enzymatically detached from the wells using 0.25% trypsin (Gibco). Viable and nonviable cells were detected by trypan blue (Sigma) and counted using a hemocytometer (Housser Scientific Co., Horsham, PA).

5.2.5. TUNEL assay

DNA strand breaks in apoptotic cells were measured by the TUNEL assay using the In-situ Detection Kit (Roche Molecular Biochemicals, Germany). Samples were fixed with 4% paraformaldehyde in PBS for 15 min and incubated in a 0.1% ice-cold Triton X-100 solution for permeabilization for 10 min according to the manufacturer's instructions. Cell were then washed 3 times with PBS and reacted with 50 µl of the TUNEL reaction mixture at 37°C for 60 min in a dark, humidified chamber. Cells were washed three times in PBS and incubated for 30 minutes with 50 µl of the Converter-POD (anti-fluorescein peroxidase) (Roche Diagnostics, Castle Hill, Australia) followed by another 15 minutes with DAB to ensure the detection of TUNEL labeled cells. Under light microscopy, the number of TUNEL-positive cells per high-power field (100x) was counted and expressed as a percentage of the total

number of cells present in that field.

5.2.6. Transfection with dominant negative akt

The kinase-inactive Akt construct (Upstate Biotechnology) functions as a dominant negative inhibitor of endogenous Akt. Dominant negative-Akt or empty vector as a control was added to DMEM without antibiotics to a final concentration of 2μg/well in 6 well plates and mixed with liopofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in a 1:1 ratio. Cells were then incubated with this Akt dominant negative DNA transfection solution for 48 hours. Successful transfection of dominant negative - Akt was confirmed by western blot analysis.

5.2.7. Scrape loading and dye transfer assay

To determine the level of gap junctional communication in human granulosa cells, a scrape loading and dye transfer assay was performed. The cell monolayer at full confluence was washed three times with PBS and scraped using a surgical blade before adding the fluorescent dye, lucifer yellow (MW 457.2 Da) as a 0.5% solution. After 3 min of incubation, the Lucifer Yellow solution was removed. The culture was rinsed three times with PBS and fixed with 4% paraformaldehyde in PBS for 15 min.

The level of dye transfer was measured as the distance from the scrape line to the line between farthest and nearest extent of lucifer yellow stained cells.

5.2.8. Connexin 43 siRNAs transfection

ON-TARGET siRNA specifically targeting Cx43 (Dharmacon, Thermo Fisher Scientific) and control siRNA (Dharmacon, Thermo Fisher Scientific) were added to DMEM without antibiotics to a final concentration of 40nM and incubated for 15 min at room temperature with the RNA transfection reagent, Lipofectamine™ RNA iMAX (Invitrogen, Carlsbad, CA, USA) in a 1:1 ratio. Cells were then incubated with this siRNA transfection solution for 48 hours. Successful knockdown of connexin 43 was confirmed by western blot analysis.

5.2.9. Statistical analysis

Results from more than three independent experiments were presented as the mean ± SD. Statistical analysis was carried out using GraphPad Prism 5.0. One-way analysis of variance (ANOVA) was used to analyse normally distributed variables, whereas non-parametric Kruskal–Wallis test was used to analyze nonnormally distributed variables. Statistical significance was defined at a P *level of* <0.05.

5.3. Results

5.3.1. Immortalized granulosa cell line expressed functional GnRH receptors

We first assessed whether our two new immortalized human granulosa cell lines (IHG 1 and 2) expressed GnRH receptors and if present, their functionality. Using LβT2 (derived from a mouse pituitary adenoma) and the human primary cultured granulosa cells from three individual IVF patients as controls, we confirmed that our immortalized cell lines indeed expressed GnRH receptors although the expression was more marked in IHG1 than IHG2 (Fig 17A).

Since GnRH activates multiple signaling pathways including ERK1/2 in human luteal-granulosa cells (288), we tested whether this kinase was activated in response to GnRH-I in our immortalized cell lines. GnRH-I induced a time-dependent increase in ERK1/2 phosphorylation in IHG1 but little change in IHG2 (Fig 17B). We therefore chose IHG1 for our current study.

5.3.2. Anti-proliferative effects of GnRH-I or II on immortalized human granulosa cells

Cells cultured with and without GnRH-I or II (10^{-7}M) in 24-well plates for 24,

48 and 72 h were counted with a hemocytometer. GnRH-I or II both inhibited granulosa cell proliferation after 48h ($P<0.05$) and 72h ($P<0.05$) treatment with no significant differences between GnRH-I or II (Fig. 18).

5.3.3. Apoptotic effects of GnRH-I or II on human granulosa cells

GnRH-I or II treatment for 72 hours significantly increased the number of apoptotic or TUNEL-positive cells (Fig.19) and increased activated caspase-3 (Fig.20) compared to controls as observed previously (208). However, GnRH-I or II did not affect Bad, Bcl-2 phosphorylation, and Bok, Bax, Bik and Bim expression (Fig.20). In separate experiments with primary cultured human granulosa cells derived from six women undergoing IVF, cleaved caspases-3 expression remained unchanged in patient 7 and 9 but increased in patients 4, 5 and 6 with GnRH-I or II treatment (Fig. 21).

5.3.4. The attenuating effects of GnRH-I or II on IGF-I in granulosa cells

We examined the effects of treatment with IGF-1 (50ng/ml), GnRH I or II (10^{-7}M) alone, or IGF-I with GnRH-I or II for 24, 38 and 72h on granulosa cells proliferation. IGF-I increased granulosa cell proliferation in a time-dependent manner, while GnRH-I or II suppressed granulosa cell proliferation which was more marked by

72h. GnRH-I or II also significantly attenuated the stimulatory effect of IGF-I on granulosa cell proliferation (Fig.22).

After 72h culture, GnRH-I or II increased ($p<0.05$) while IGF-I decreased ($p<0.05$) the number of TUNEL-positive cells compared to controls (Fig. 23). GnRH-I or II treatment also attenuated the anti-apoptotic effects of IGF-I (Fig. 23). Correspondingly, IGF-I decreased while GnRH-I or II increased cleaved caspases-3 in our IHG1 cells; furthermore, GnRH-I or II attenuated the suppressive effect of IGF-I on the caspase-3 activities (Fig. 24). We further assessed if these effects were similarly observed in primary cultured granulosa cell from the 8 patients undergoing IVF. IGF-I reduced whereas GnRH-I or II increased caspase-3 activities and attenuated the suppressive effect of IGF-I on caspase-3 in patients 4, 6, and 8 but minimally in patients 10 and 11 (Fig 25).

5.3.5. Effects of GnRH-I or II on IGF-I induced akt signaling pathways

We examined IGF-I receptor phosphorylation as short-term changes following GnRH-I or II (10^{-7}m) alone, IGF-I (50 ng/ml) alone or IGF-I with GnRH-I or II up to 60 min, and total IGF-I expression as longer term changes at 72h by western blot analysis. GnRH-I or II did not affect IGF-I receptor phosphorylation at all

time intervals (5, 10, 30 and 60 min) (Fig. 26, upper and middle panel). IGF-I induced IGF-I receptor phosphorylation at 15 min, whereas GnRH-I or II did not affect basal or IGF-I induced receptor phosphorylation (Fig. 26, lower panel). As expected, IGF-I decreased IGF-I receptor levels by ～50 - 60%. In contrast, GnRH-I or II did not affect basal IGF-I receptor expression nor corresponding changes induced by IGF-I (Fig. 27).

Therefore, we examined Akt (downstream signaling molecule of IGF-I receptor) phosphorylation following GnRH-I or II (10^{-7}M) alone, IGF-I (50 ng/ml) alone or IGF-I with GnRH-I or II for 15 min by western blot analysis. IGF-I induced phosphorylation of Akt (Ser-473) at 15 min as expected. In contrast, GnRH-I or II did not affect basal Akt (Ser-473) phosphorylation but down-regulated IGF-I induced Akt phosphorylation at 15 min (Fig. 28). These inhibitory effects were attenuated by co-treatment with antide (Fig.29).

The involvement of Akt in the interactions of IGF-I and GnRH-I or II in regulating granulosa cell apoptosis was demonstrated by reversal of the inhibitory effects of IGF-I on GnRH-I or II induced cleaved caspase-3 activities. Firstly, both dominant negative Akt transfection (which inhibits functional activities of Akt) (Fig.30) and pretreatment of Wortmannin, an inhibitor of PI3K (upstream of Akt)

(Fig.32) reversed the inhibitory effects of IGF-I on cleaved caspase-3 activities.

Secondly, these results were further demonstrated by dominant negative Akt transfection or Wortmannin pretreatment with GnRH-I or II (10^{-7}M) alone, IGF-I (50 ng/ml) alone, or IGF-I with GnRH-I or II. The interactions of IGF-I and GnRH-I or II in regulating cleaved caspase-3 activities in human granulosa cells were attenuated by dominant negative Akt transfection (Fig.31) or Wortmannin pretreatment (Fig.33).

These findings suggest that the interactions of IGF-I and GnRH-I or II in regulating granulosa cell apoptosis are mediated through Akt signaling and not IGF-I receptor levels in human granulosa cells.

5.3.6. Effects of GnRH-I or II on gap junctional communication in granulosa cells

Following scrape loading and dye transfer assay, lucifer yellow (the gap junction permeable fluorescent dye) diffused widely into the neighboring cells in controls but not cells treated with GnRH-I or II (10^{-7}M). When cells were first pre-incubated with antide for 1h before GnRH-I or II treatment, Lucifer yellow was noted to diffuse widely into neighboring cells (Fig.34).

GnRH-I or II induced Cx43 phosphorylation at 72h. In contrast, GnRH-I or II did not affect Cx43 phosphorylation at 15 min or total Cx43 protein levels at both

15min and 72h (Fig.35). As expected, antide attenuated GnRH-I or II induced Cx43 phosphorylation at 72h (Fig. 35). After almost complete knockdown of Cx43 with 40 nM of target siRNA at 48h, cleaved caspases-3 expression remained unchanged compared to those of control siRNA (Fig. 36).

The knockdown of Cx43 did not affect GnRH-I or II induced cleaved caspases-3 activities at 72h (Fig. 37). These results suggest that the inhibitory effects of GnRH-I or II on lucifer yellow transfer in human granulosa cells are not mediated through Cx43-related gap junctional changes but are concurrent with GnRH-I or II induced granulosa cell apoptosis at 72h.

5.4. Discussion

We previously reported that GnRH-I or II induced apoptotic effects in human granulosa cells through the caspase pathway using the SVOG human granulosa cell line as the research model (208). We confirmed that the new cell line (IHG1) in our current study expressed GnRH receptors and ERK1/2 activities. Our current study not only confirmed the involvement of the caspase-dependent extrinsic pathway in GnRH-I or II induced apoptosis in our new granulosa cell line (IHG1), but also in primary cultured human granulosa cells.

In contrast, we have found no significant changes in the expression or

phosphorylation of Bad, Bcl-2, Bok, Bax, Bik and Bim following GnRH-I or II treatment in our study. These results suggest that the apoptotic effects of GnRH-I or II on human granulosa cells are mediated through the caspase-dependent extrinsic pathway rather than the intrinsic pathway involving Bcl-2 family members. On the other hand, the apoptotic effects of GnRH agonist on rat granulosa cells involve some of the Bcl-2 family members (291) and caspase-3 (316). At present, it is uncertain if these differences are species-specific, or due to variation in experimental conditions, and/or the effects of SV40 large T antigen transfection in establishing cell lines (277, 278), Further studies are needed to assess the effects of GnRH-I or II on Bcl-2 family members in primary cultured human granulosa cells to corroborate our findings in our IHG1 cell line.

Another important finding of this study is that GnRH-I or II can exert its apoptotic effects by interfering with IGF-I activities. However, instead of exerting its effects on IGF-I receptor itself, GnRH-I or II affects the downstream signaling molecules of IGF-I receptor such as Akt. These findings are in contrast with studies showing that GnRH agonists can decrease IGF-I receptor phosphorylation (241) and expression (241) in androgen-independent prostate cancer cells. Therefore, the interactions between GnRH and IGF-I receptor are still controversial and remain to be

established.

We have further confirmed that GnRH-I or II could counteract the inhibitory effect of IGF-I on apoptosis in primary cultured human granulosa cells from five of the eight patients in which GnRH-I or II attenuated the inhibitory effects of IGF-I on caspase-3 activities. That these findings were not observed in all patients might reflect the heterogeneity of the granulosa cell population, and likely differences in patient characteristics and/or ovarian responses to IVF treatment protocols. Taken together, these results are consistent with involvement of Akt activation in granulosa cell survival in pigs (317) and chicken (193). Furthermore, not only have we confirmed the role of Akt in our granulosa cell models in regulating apoptosis, we have demonstrated, for the first time, that GnRH-I or II can counteract the anti-apoptotic effects of IGF-I on human granulosa cells by inhibiting Akt activation.

In conclusion, GnRH-I or II induces apoptosis in our human granulosa cell models through caspase-3 dependent extrinsic pathway, rather than bcl-2 family dependent intrinsic pathway, and attenuates IGF-I actions through Akt. Cx43 induced gap junctional changes are not directly involved in initiating granulosa cell apoptosis but are concurrent with apoptosis induced by GnRH-I or II. Further studies are needed to further validate our findings in granulosa cells from unstimulated human ovaries.

However, until these cells are easily accessible for research, findings from our study have provided interesting hypotheses on the regulation of GnRH-I or II induced apoptosis and its interactions with growth factors, such as IGF-I, and gap junctional communication in human granulosa cells.

5.5. Figures

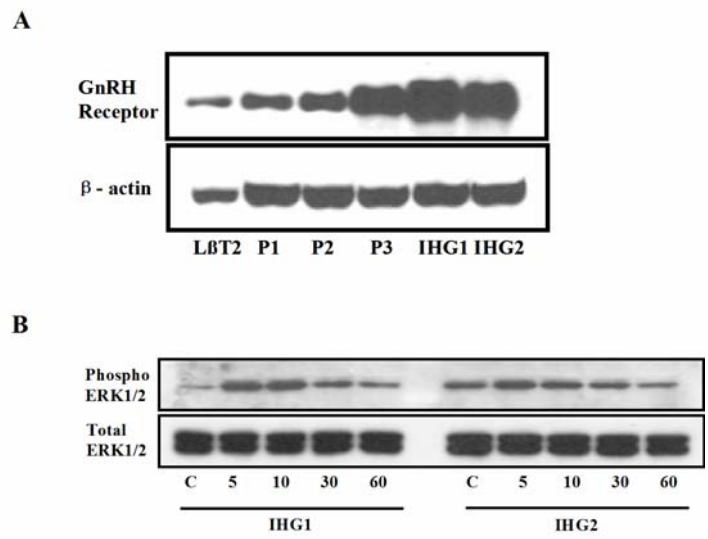

Figure 17. GnRH receptor expression and effects of GnRH-I induced ERK1/2 phosphorylation in immortalized granulosa cell lines. (A) GnRH receptor expression was confirmed by western blotting in the two immortalized human granulosa cell lines (IHG1 and IHG2) as well as granulosa cells from primary cultures in three patients (designated by P1, 2, and 3). Mouse pituitary adenoma cell lines (LβT2) and β-actin were the positive and internal controls respectively.(B) Corresponding phosphorylation of ERK1/2 ("Phospho-ERK1/2") in control ("C") and following GnRH-I treatment for up to 60 min were assessed by western blot analysis. Total ERK1/2 and β-actin were the internal control.

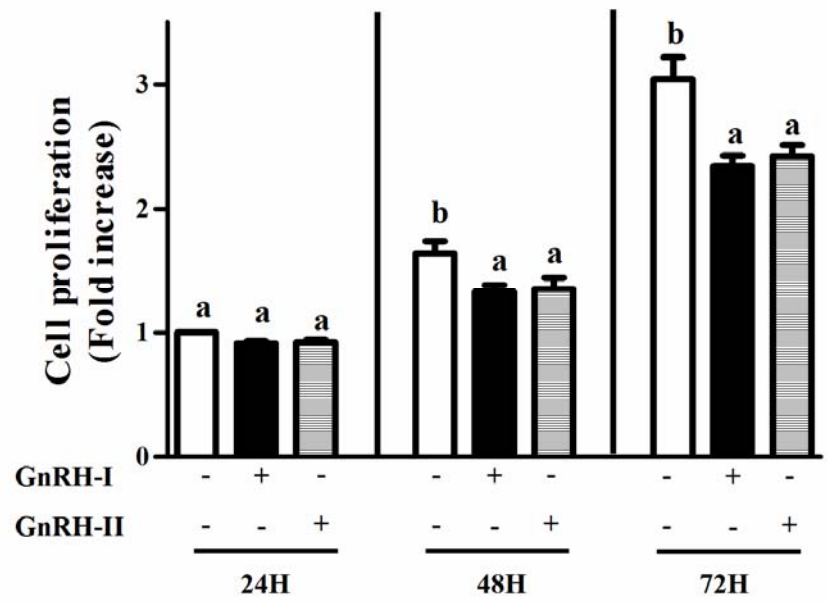

Figure 18. Anti-proliferative effects of GnRH-I or II on human granulosa cells. Cell proliferation levels were evaluated by direct cell counting after IHG1 cells were cultured with and without GnRH-I or II (10^{-7}M) for 24, 48 and 72h. GnRH-I or II significantly inhibited granulosa cell proliferation after 48 and 72 hours treatment with no significant differences between GnRH-I or II. Results from three independent experiments were presented as the mean ± SD. Means without a *common letter* are significantly different ($P < 0.05$).

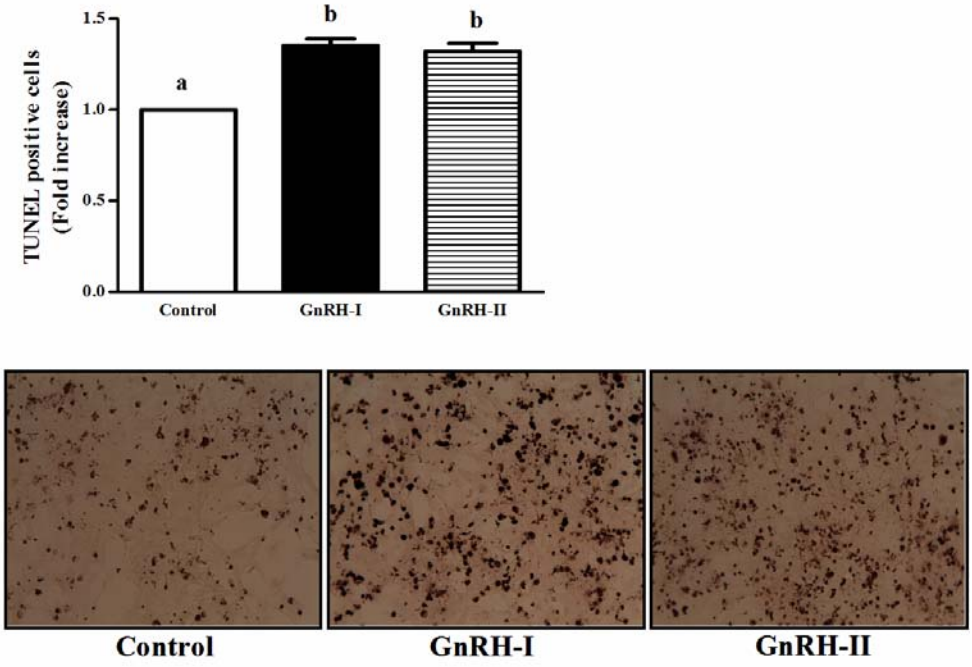

Figure 19. Apoptotic effects of GnRH-I or II on human granulosa cells. Cell apoptosis levels were assessed with the TUNEL assay after treatment with and without GnRH-I or II (10^{-7}M). TUNEL-positive cells were characterized by densely stained circular bodies representing fragmented DNAs of apoptosis. GnRH-I or II treatment of IHG1 cells increased the number of TUNEL-positive cells at 72 h. Results from three independent experiments were presented as the mean ± SD. Means without a *common letter* are significantly different ($P < 0.05$).

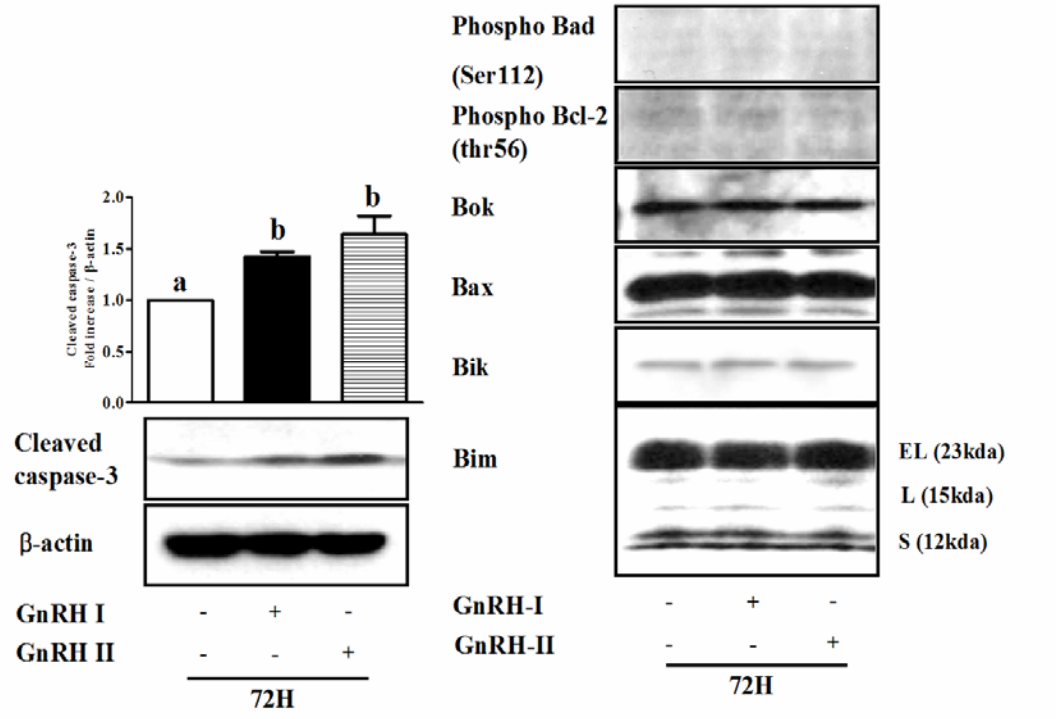

Figure 20. Apoptotic effects of GnRH-I or II on human granulosa cells mediated through caspase-dependant extrinsic pathway. Cell apoptosis levels were assessed with western blotting using antibodies recognizing cleaved caspase-3, Bad, Bcl-2, Bok, Bax, Bik and Bim after treatment with and without GnRH-I or II (10^{-7}M). GnRH-I or II increased cleaved caspase-3 expression at 72 h (left panel) but had no effects on Bad, Bcl-2 phosphorylation, and Bok, Bax, Bik and Bim expression (right panel). Means without a *common letter* are significantly different ($P < 0.05$).

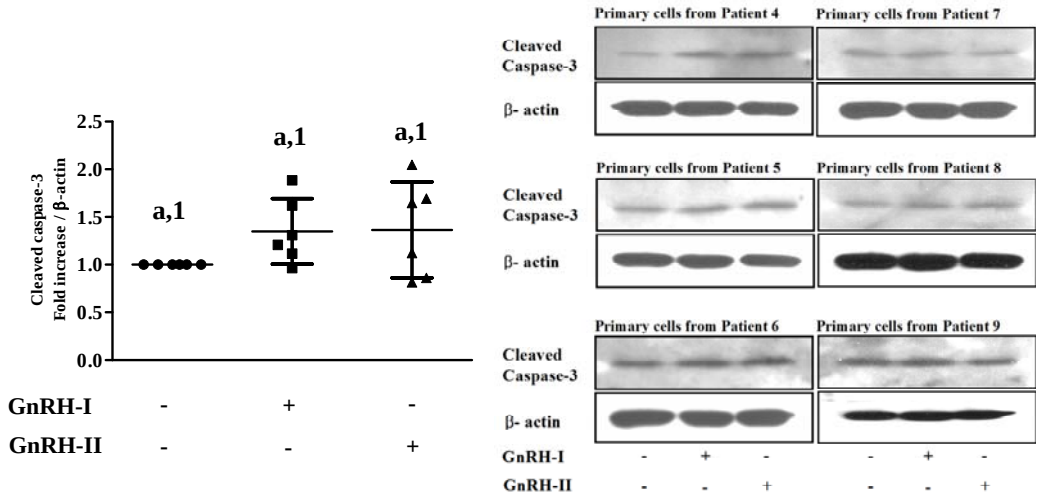

Figure 21. Apoptotic effects of GnRH-I or II on primary cultured human granulosa cells. The expression of cleaved caspase-3 in granulosa cells from primary cell cultures of six patients was assessed by western blotting for comparison. After pre-culture for 24h, GnRH-I or II increased cleaved caspase-3 expression in patient 4, 5, 6 and 8 at 72h as in IHG1 cells but unchanged in patient 7 and 9. Scatter plot depicting individual data points of cleaved caspase-3 expressions derived from primary cultured granulosa cells stimulated with GnRH-I or II. Results from six different patients were presented as the mean ± SD. Means without a *common letter* (a,b: One-way ANOVA and 1,2: Non-parametric Kruskal–Wallis test) are significantly different ($P < 0.05$).

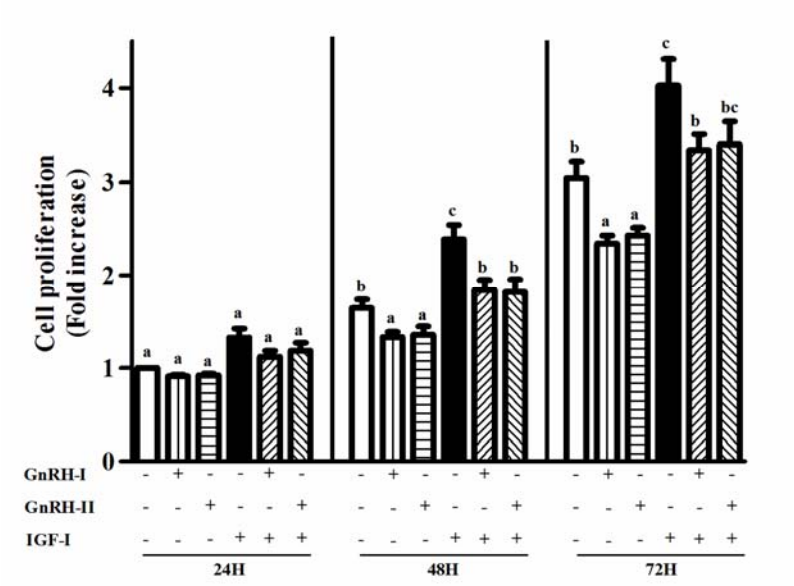

Figure 22. GnRH-I or II exert anti-proliferative effects on human granulosa cells by interfering with IGF-I activity. Cell proliferation levels were evaluated by direct cell counting after treatment with and without GnRH-I or II (10^{-7}M). IHG1 cells were cultured with IGF-I (50 ng/ml) alone, GnRH-I or II (10^{-7}M) alone, or IGF-I with GnRH-I or II for 24, 48 and 72h. IGF-I increased cell proliferation, while GnRH-I or II had opposite effects correspondingly. Co-treatment with GnRH-I or II also attenuated the above effects of IGF-I. Results from three independent experiments were presented as the mean ± SD. Means without a common letter are significantly different ($P < 0.05$).

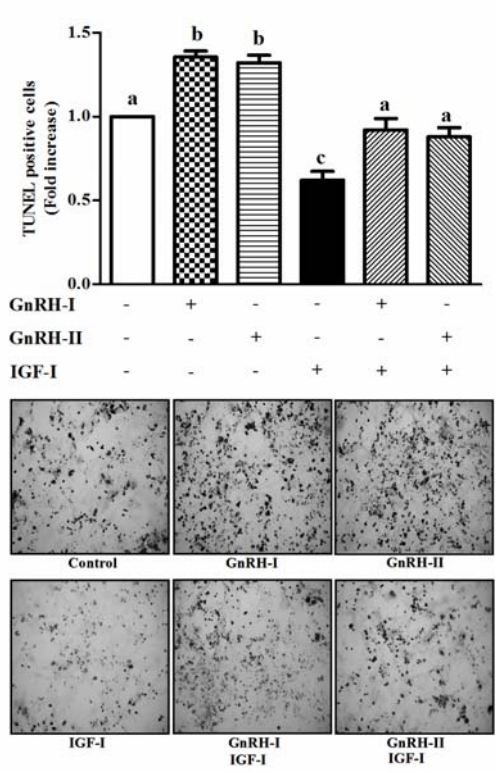

Figure 23. The attenuating effects of GnRH-I or II on IGF-I activities in granulosa cells apoptosis. Cell apoptosis levels were assessed with the TUNEL assay after treatment with and without GnRH-I or II (10^{-7}M). IHG1 cells were cultured with IGF-I (50 ng/ml) alone, GnRH-I or II (10^{-7}M) alone, or IGF-I with GnRH-I or II for 72h. IGF-I decreased TUNEL-positive cell numbers while GnRH-I or II had opposite effects correspondingly. Co-treatment with GnRH-I or II also attenuated the above effects of IGF-I. Results from three independent experiments were presented as the mean ± SD. Means without a common letter are significantly different ($P < 0.05$).

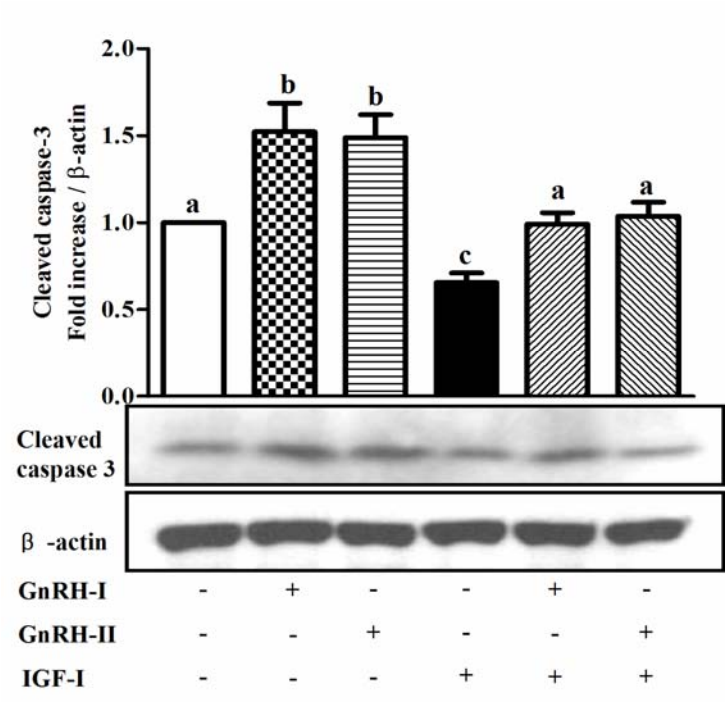

Figure 24. The attenuating effects of GnRH-I or II on the anti-apoptotic activities of IGF-I in granulosa cells apoptosis. Apoptosis levels were assessed with the western blotting after treatment with and without GnRH-I or II (10^{-7}M). IHG1 cells were cultured with IGF-I (50 ng/ml) alone, GnRH-I or II (10^{-7}M) alone, or IGF-I with GnRH-I or II for 72h. IGF-I decreased while GnRH-I or II increased cleaved caspases-3 in our IHG1 cells; furthermore, GnRH-I or II significantly attenuated the expression of cleaved caspase 3 induced by IGF-I. Results from three independent experiments were presented as the mean ± SD. Means without a common letter are significantly different ($P < 0.05$).

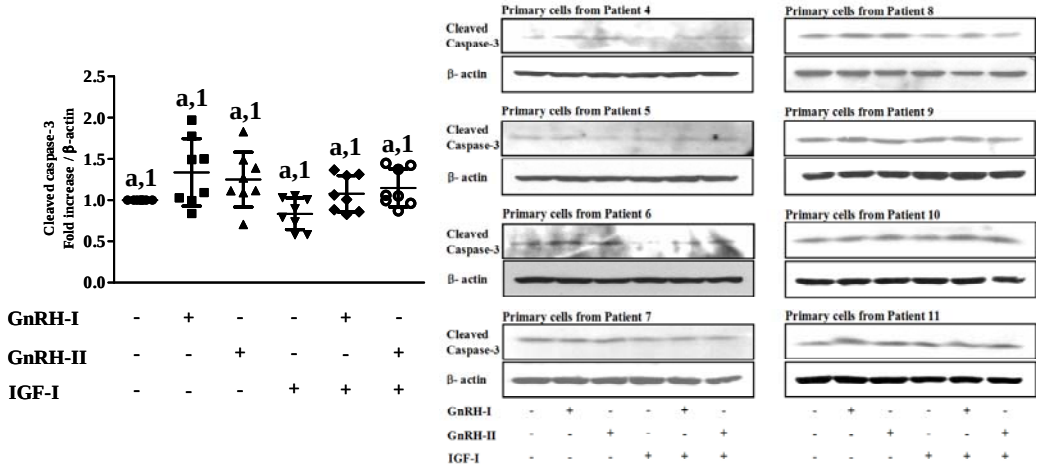

Figure 25. The attenuating effects of GnRH-I or II on the anti-apoptotic activities of IGF-I in primary cultured granulosa cells. The corresponding expression of cleaved caspase-3 in granulosa cells from primary cell cultures of eight patients was assessed by western blotting for comparison. After pre-culture for 24h, IGF-I alone, GnRH-I or II alone, or IGF-I with GnRH-I or II exerted similar effects on cleaved caspase-3 expression at 72h as in IHG1 cells in patient 4, 6 and 8 but minimal (patient 10 and 11) or no changes (patient 5, 7 and 9) in five of the eight patients. Scatter plot depicting individual data points of cleaved caspase-3 expressions derived from primary cultured granulosa cells stimulated with GnRH-I or II alone, IGF-I alone or IGF-I with GnRH-I or II. Results from eight different patients were presented as the mean ± SD. Means without a *common letter* (a,b: One-way ANOVA and 1,2: Non-parametric Kruskal–Wallis test) are significantly different ($P < 0.05$).

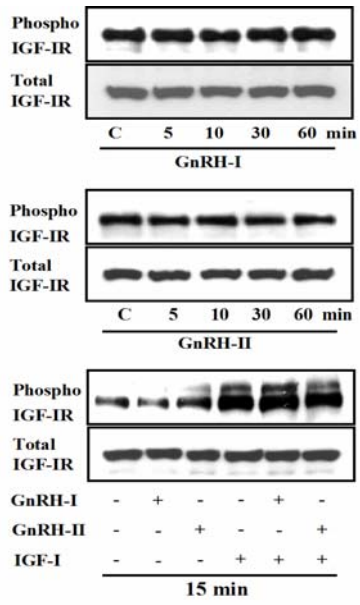

Figure 26. GnRH-I or II treatment did not affect phosphorylation of IGF-I receptor. IGF-I receptor phosphorylation ("Phospho-IGF-IR") were assessed by western blotting. After pre-culture for 24h, IHG1 cells were cultured with IGF-I (50 ng/ml) alone, GnRH-I or II (10^{-7}M) alone, or IGF-I with GnRH-I or II. GnRH-I or II treatment had no effects on IGF-I receptor phosphorylation and had no effects on increased IGF-I receptor phosphorylation induced by IGF-I treatment. Results from three independent experiments were presented as the mean ± SD. Means without a *common letter* are significantly different ($P < 0.05$). "C" denotes no treatment; Total IGF-IR was the internal control.

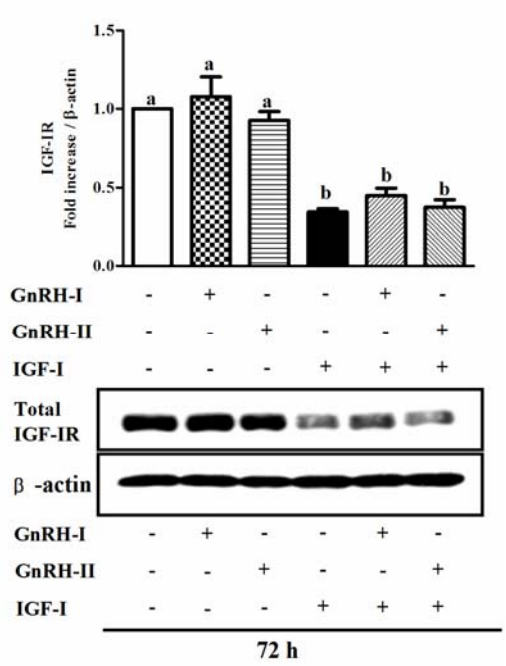

Figure 27. GnRH-I or II treatment did not affect IGF-I receptor expression itself. IGF-I receptor expression ("Total IGF-IR") were assessed by western blotting. After pre-culture for 24h, IHG1 cells were cultured with IGF-I (50 ng/ml) alone, GnRH-I or II (10^{-7}M) alone, or IGF-I with GnRH-I or II. GnRH-I or II treatment had no effects on IGF-I receptor protein levels and had no effects on decreased IGF-I receptor protein levels induced by IGF-I. Results from three independent experiments were presented as the mean ± SD. Means without a common letter are significantly different ($P < 0.05$).

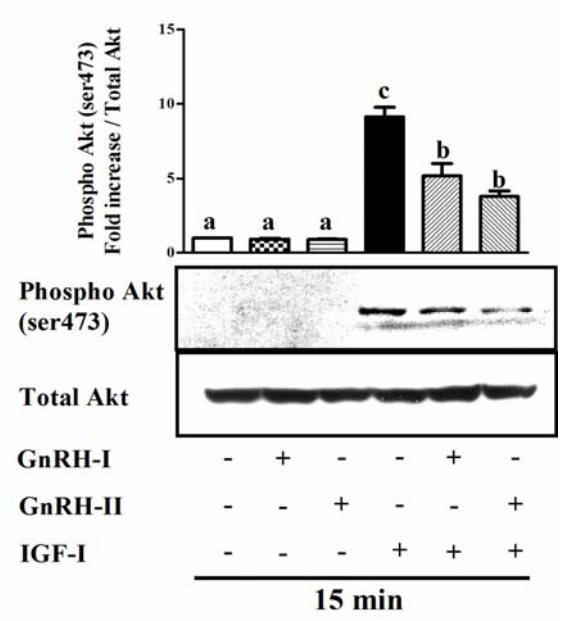

Figure 28. GnRH-I or II interfere with IGF-I induced Akt signaling pathways. Phosphorylation of Akt (Ser-473) ("Phospho Akt") was assessed by western blotting. After pre-culture for 24h, IHG1 cells were cultured with IGF-I (50 ng/ml) alone, GnRH-I or II (10^{-7}M) alone, or IGF-I and GnRH-I or II for 15 min. GnRH-I or II had no effects on phosphorylation of Akt but down-regulated corresponding phosphorylation induced by IGF-I treatment. Results from three independent experiments were presented as the mean ± SD. Means without a common letter are significantly different ($P < 0.05$).

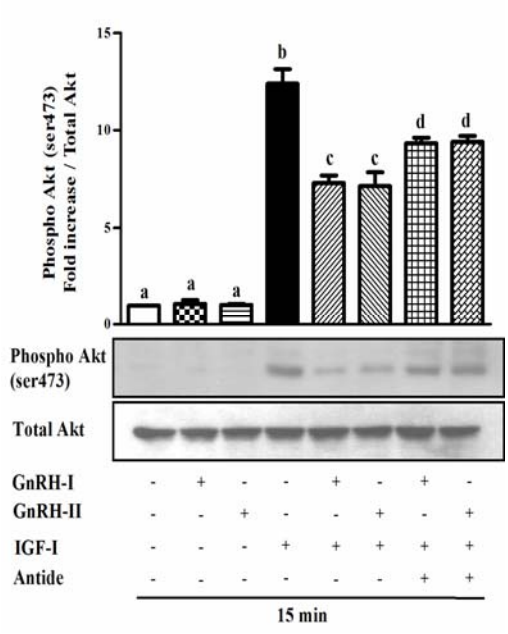

Figure 29. GnRH-I or II interfere with IGF-I induced Akt signaling pathways. Phosphorylation of Akt (Ser-473) ("Phospho Akt") was assessed by western blotting. After pre-culture for 24h, IHG1 cells were cultured with IGF-I (50 ng/ml) alone, GnRH-I or II (10^{-7}M) alone, or IGF-I and GnRH-I or II for 15 min. IGF-I induced phosphorylation of Akt (Ser-473) and GnRH-I or II led to down-regulation of IGF-I-induced Akt phosphorylation. These inhibitory effects of GnRH-I or II on IGF-I-mediated Akt phosphorylation were attenuated by co-treatment with GnRH specific antagonist, antide. Results from three independent experiments were presented as the mean ± SD. Means without a common letter are significantly different ($P < 0.05$).

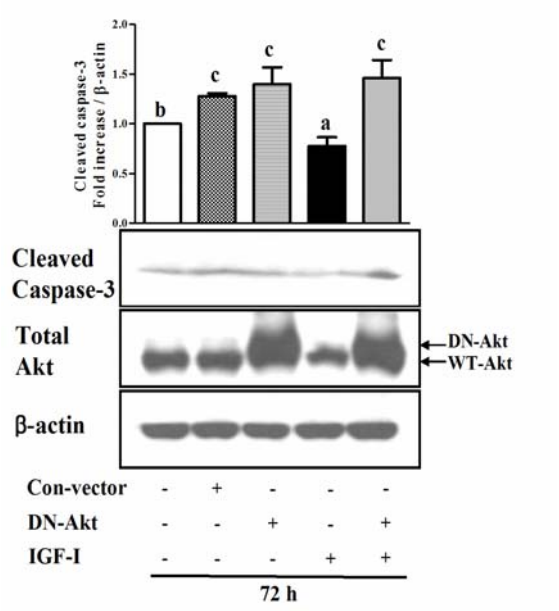

Figure 30. The effects of dominant negative Akt ("DN-Akt") on cleaved caspase-3 expression. Cleaved caspase-3 expression was assessed by western blotting. After pre-culture for 24h, IHG1 cells were transfected with 2μg DN-Akt for 48h. The inhibitory effect of IGF-I on cleaved caspase-3 expression was reduced following dominant negative Akt transfection. Results from three independent experiments were presented as the mean ± SD. Means without a common letter are significantly different ($P < 0.05$).

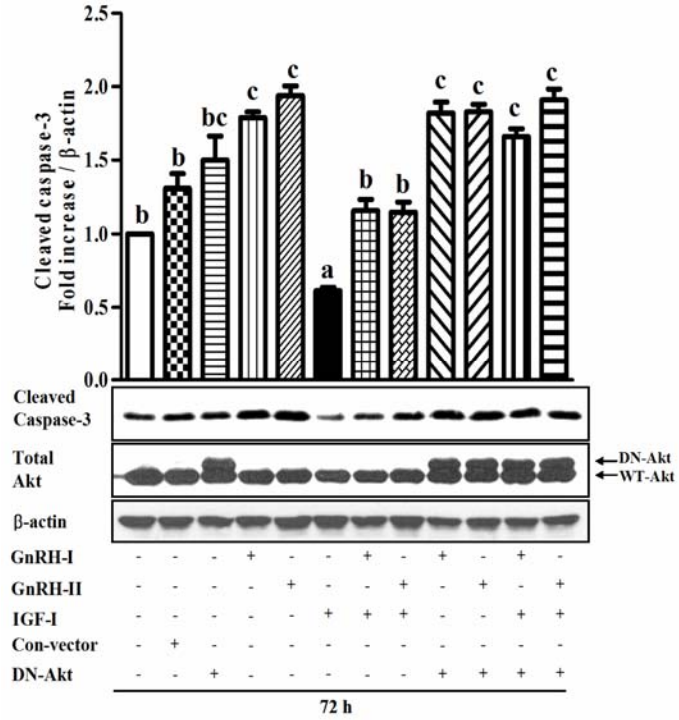

Figure 31. The inhibitory effects of IGF-I on GnRH-I or II induced caspase-3 activation were reduced by dominant negative Akt transfection. After pre-culture for 24h, IHG1 cells were transfected with 2μg M DN-Akt for 48h before treatment with GnRH-I or II alone, IGF-I alone, or GnRH- I or II and IGF-I for 72h. The inhibitory effects of IGF-I on GnRH-I or II induced cleaved caspase-3 expression was reduced following dominant negative Akt transfection. Results from three independent experiments were presented as the mean ± SD. Means without a *common letter* are significantly different ($P < 0.05$).

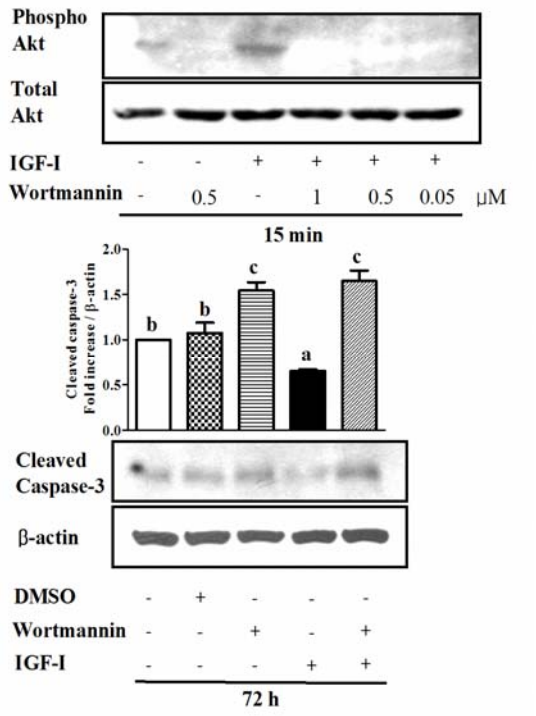

Figure 32. The effects of Wortmannin which inhibits PI3K (upstream target of Akt) on cleaved caspase-3 expression. After pre-culture for 24h, IHG1 cells were pretreated with 0.05, 0.5 and 1μM Wortmannin for 1h before treatment with IGF-I for 15min and 72h. Phosphorylation of Akt (Ser-473)("Phospho Akt) and cleaved caspase-3 was assessed by western blotting. Treatment with 0.05 μM Wortmannin completely suppressed IGF-I-induced Akt phosphorylation at 15min. The inhibitory effects of IGF-I on cleaved caspase-3 expression were reduced following wortmannin treatment. Results from three independent experiments were presented as the mean ± SD. Means without a *common letter* are significantly different ($P < 0.05$).

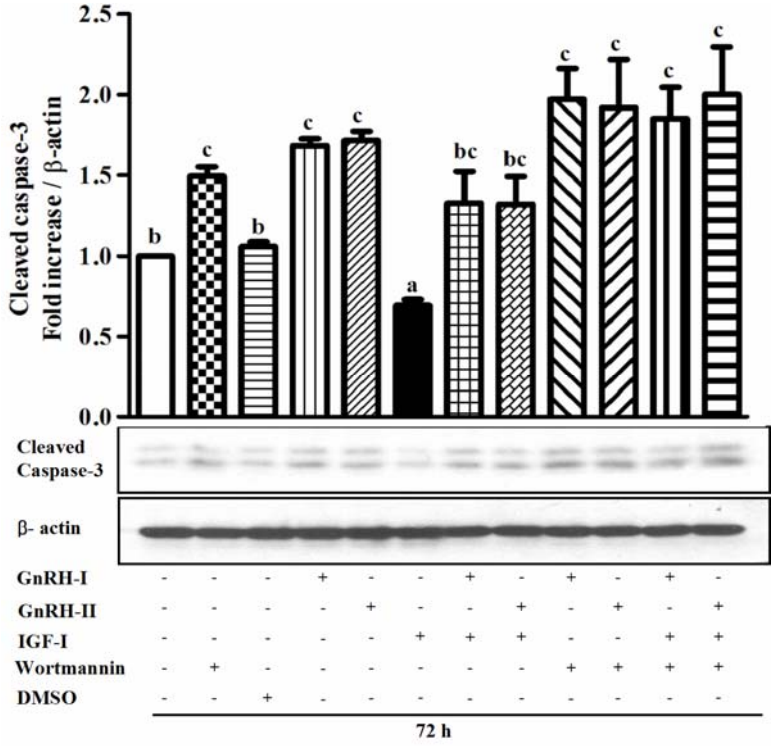

Figure 33. The inhibitory effects of IGF-I on GnRH-I or II induced caspase-3 activation were reduced by Wortmannin treatment. After pre-culture for 24h, IHG1 cells were pretreated with 0.05μM Wortmannin for 1h before treatment with GnRH-I or II alone, IGF-I alone, or GnRH- I or II and IGF-I for 72h. The inhibitory effects of IGF-I on GnRH-I or II induced cleaved caspase-3 expression were reduced following wortmannin treatment. Results from three independent experiments were presented as the mean ± SD. Means without a *common letter* are significantly different ($P < 0.05$).

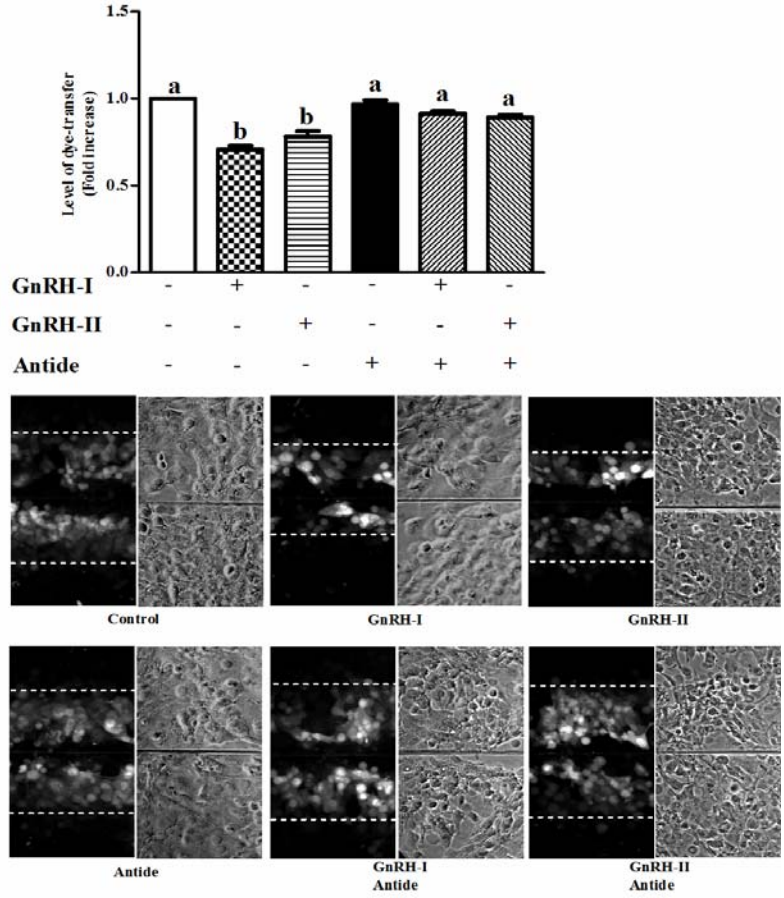

Figure 34. The effects of GnRH-I or II on gap junctional communication in human granulosa cells. Scrape loading and dye transfer assay. IHG1 cells were treated with GnRH-I or II (10^{-7}M) or together with antide (10^{-7} M) for 72h. The level of gap junctional communication was represented by the distance of dye migration on fluorescence microscopy on SL/DT assay. GnRH-I or II inhibited lucifer yellow transfer, effects that were attenuated by co-culture with antide. Results from three independent experiments were presented as the mean ± SD. Means without a common letter are significantly different ($P < 0.05$).

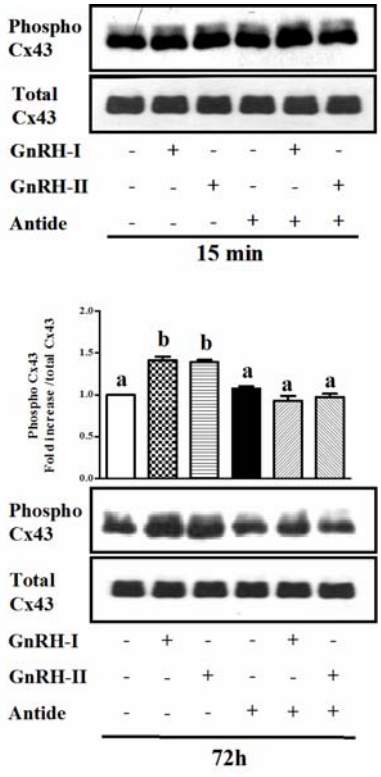

Figure 35. Long-term exposure of GnRH-I or II induced Cx43 serin368 phosphorylation. Phosphorylation of connexin 43 ("Phospho-Cx43"), which inhibits functional activities of Cx43, was assessed by western blot analysis. After pre-culture for 24h, IHG1 cells were cultured with GnRH-I or II (10^{-7}M) with and without antide (10^{-7} M) for 15 min or 72h. GnRH-I or II increased Cx43 phosphorylation at 72h (but not 15 min), effects that were attenuated by antide.

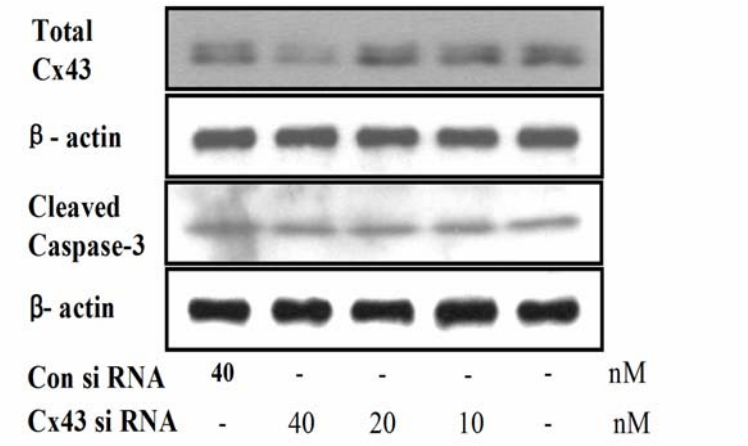

Figure 36. The effects of Cx43 siRNA transfection on cleaved caspase-3 expression. After preculture for 24h, IHG1 cells were transfected with 10, 20 and 40 nM of Cx43 siRNA for 48h. Cx43 and cleaved caspase-3 expression was assessed by western blot analysis. Transfection of 40nM Cx43 siRNA almost completely suppressed Cx43 expression but had no effects on cleaved caspases-3 expression.

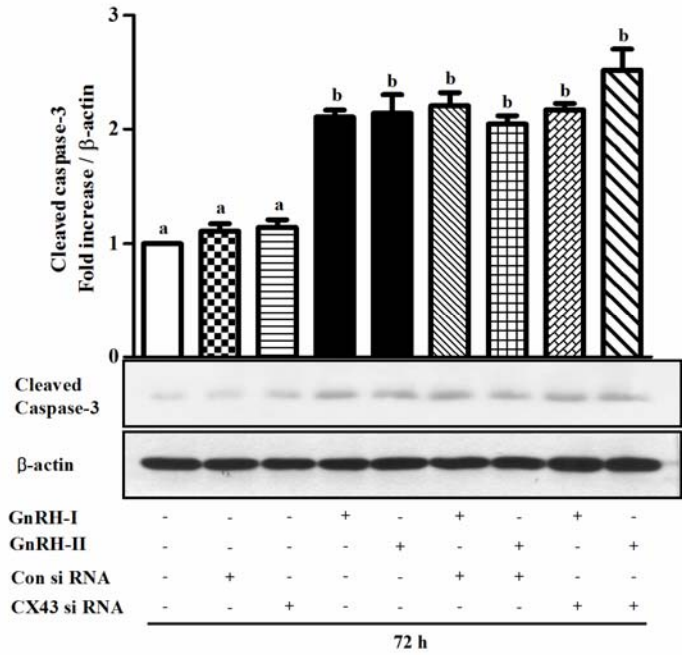

Figure 37. The inhibitory effects of GnRH-I or II on gap junction in human granulosa cells were not mediated through the Cx43-related gap junction. Cleaved caspase-3 expression was assessed by western blot analysis. Transfection of 40nM Cx43 siRNA had no effects on cleaved caspases-3 expression nor GnRH-I or II induced cleaved caspases-3 expression. Results from three independent experiments were presented as the mean ± SD. Means without a *common letter* are significantly different ($P < 0.05$).

6. Summary and future studies

6.1. Summary

Although some of the molecular features of granulosa cells have been characterized, related studies are usually confined to animals and granulosa tumor cells or a limited set of factors are studied in normal human granulosa cells. Thus, a combined profile providing a more comprehensive appreciation of the human granulosa cell is lacking. In this study, we have performed integrated investigations of multiple characteristics (such as morphology, anchorage-independent growth, growth potential, endocrine function and multiple molecular profiles) that play important roles in the structure and function of ovarian granulosa cells, in order to elucidate more clearly the characteristics of human ovarian granulosa cells. Although there were some biological variations among patients, this study is the first to report multiple cellular and molecular profiles for primary cultured human granulosa cells and these established characteristics of normal granulosa cells are helpful to a better understanding of follicular function and development.

The studies for the regulation of granulosa cell function are commonly performed in primary cultured granulosa cells obtained from *in vitro* fertilization (IVF) procedures, because of their availability. However, human granulosa cells isolated

from IVF procedures have limited proliferative capacity and can rarely be cultured for longer than a few days. These cells are usually pooled for culture and are therefore not homogeneous, and consequently, experimental results can vary between studies. Stable immortalized granulosa cell lines are additional research tools that can be used to investigate some of the cellular and molecular characteristics of human granulosa cells. Therefore, we have established two steroidogenic granulosa cell lines and determined their characteristics. Although there were some differential expression patterns of several molecular profiles between primary cultured and immortalized cell lines, perhaps because of effects of the SV40 large T antigen on normal physiological cell functions, various characteristics considered essential for normal granulosa cell functions were present in our newly established cell lines (Table 2). Therefore, our well-characterized cell line systems can be a valuable source to study the potential mechanisms of human follicular development and differentiation in the future.

The direct effects of GnRH on granulosa cell apoptosis have been investigated by several researchers and have been shown to increase the incidence of apoptosis in animal granulosa cells and down-regulate the proliferation of human granulosa cells. Because of their close association with the oocyte, increased granulosa cell apoptosis may influence the development and quality of the oocyte, increase the occurrence of

empty follicles, poor oocyte fertilization, and result in lower pregnancy rates. However, the direct effects of GnRH-I or II on human granulosa cell apoptosis and the underlying mechanisms involved remain unknown.

This is the first study to demonstrate that in addition to its well-established actions on the pituitary–gonadal axis, GnRH-I or II induces anti-proliferative effects through several different mechanisms in human granulosa cells. Firstly, GnRH-I or II can directly induce apoptosis in human granulosa cells. The direct apoptotic effects of GnRH-I or II are mediated by GnRH-I receptors. The intracellular signals of apoptosis induced by GnRH-I or II, in turn, are mediated by activation of the caspase-dependent extrinsic pathway, involving caspase-8, -3 and -7, rather than Bcl-2 family-dependent intrinsic pathway. In addition, FSH protects human granulosa cells from undergoing apoptosis induced by GnRH-I or II (Fig. 38)

Secondly, GnRH-I or II suppressed human granulosa cells proliferation and these anti-proliferative effects are mediated by interfering with the mitogenic activity of insulin-like growth factor-I (IGF-I) through the Akt signaling pathway. IGF-I is known to stimulate the proliferation of human granulosa cells and increase steroid secretion and responsiveness to gonadotropins in human ovarian follicles. This study provides a novel insight into the regulatory roles and signaling mechanisms mediated

by GnRH and IGF-I in human granulosa cells (Fig. 39).

During growth and development of human ovarian follicles, only a limited number of follicles proceed to the ovulatory stage, whereas more than 99% of follicles undergo the apoptotic process of atresia. The majority of atresia takes place between the pre-antral and antral stages of follicular development, when follicular growth is dependent upon gonadotropins (FSH and LH) and/or growth factors (i.e., IGF-I and EGF). Despite numerous researches on follicular atresia, the detailed biological mechanisms that determine follicular atresia in human are still unclear. The present study suggests that locally produced GnRH-I or II, via GnRH-I receptor, might be involved in controlling atresia during follicular development by interfering with the function of gonadotropin (FSH) and/or growth factors (i.e., IGF-I).

We are aware that the *in vitro* model system used in this study has some limitations. Firstly, we have further investigated that GnRH-I or II could counteract the inhibitory effect of IGF-I on apoptosis in primary cultured human granulosa cells from different IVF patients. That these findings were not observed in all patients might reflect the heterogeneity of the granulosa cell population, and likely differences in patient characteristics and/or ovarian responses to IVF treatment protocols.

Secondly, most of the atretic process takes place between the pre-antral and

early antral stages of follicular development under normal physiological conditions, whereas the primary cultured granulosa cells and the established cell lines used in this study were obtained from follicles after ovulation during the IVF procedure. However, to date no *in vivo* or *in vitro* model system for the human follicular atresia exists in which all pre-antral and antral follicles undergo atresia according to normal physiological conditions. Thus, our *in vitro* model system for the follicular atresia is not perfect, but it can be one of the possible model systems to study follicular atresia during human follicular development.

In conclusion, we have established two granulosa cell lines and performed integrated investigations of multiple characteristics to elucidate more clearly the characteristics of human ovarian granulosa cells. Additionally, the present study demonstrated that GnRH-I or II mediates anti-proliferate effects via stimulation of granulosa cell apoptosis and inhibition of IGF-I activities through Akt signaling pathway in human granulosa cells. These findings suggest that local GnRH-I or II maybe involved in the regulation of human follicular development.

6.2. Future studies

1. Although two possible molecular mechanisms (caspase-dependent apoptosis and attenuation of IGF-I function) for the GnRH-I- or II-induced anti-proliferative effect on human granulosa cells were investigated in this study, further experiments still need to be done to determine whether GnRH-I or II inhibit granulosa cell proliferation by other mechanisms such as inhibiting cell cycle arrest and decreasing progesterone production.

1) Future research will elucidate the regulatory role of GnRH-I or II in the cell cycle in human granulosa cells. Although the involvement of a GnRH agonist in human granulosa cell cycle kinetics has been shown previously (318), there is currently no information on whether GnRH-I or II can inhibit granulosa cell proliferation by regulating the cell cycle. Therefore, future experiments need to be carried out to elucidate a detailed mechanism for GnRH-I- or II-mediated cycle arrest in human granulosa cells.

2) The regulatory role of GnRH-I or II in progesterone production in human granulosa cells.

Although several studies have reported the inhibitory effects of GnRH agonists on progesterone production in primate (319) and human (320) granulosa cells, the direct effects of GnRH-I or II on steroidogenesis in human granulosa cells are still controversial. To determine whether GnRH-I or II exert their anti-proliferative effects on granulosa cells through the regulation of progesterone production, progesterone production and the expression of various steroidogenic enzymes (StAR, P450scc and 3β-HSD) needs to be assessed after treatment with GnRH-I or II, respectively.

2. In this study, we demonstrated that the apoptotic effects of GnRH-I or II might be mediated through a caspase-dependent extrinsic pathway, rather than a Bcl-2 family-dependent intrinsic pathway, in human granulosa cells. However, further experiments need to be conducted to clarify whether GnRH-I or II trigger Bcl-2 family-dependent intrinsic apoptotic pathways in human granulosa cells.

1) Involvement of the Fas ligand and Fas system should be evaluated to explain molecular mechanisms for GnRH-I or II-induced human granulosa cell

apoptosis.

3. According to our preliminary results, GnRH-I or II induced phosphorylation of PKC zeta but not of the other PKC isoforms. Therefore, it is possible to hypothesize that GnRH-I or II may exert their effects through PKC zeta in human granulosa cells. Therefore, further evaluation will be necessary to determine whether PKC zeta is involved in GnRH-I- or II-induced anti-proliferative effects and apoptosis in human granulosa cells.

Table 2 The characteristics of primary cultured granulosa cells and immortalized granulosa cell lines.

		Primary cells	IHG 1	IHG 2
Cytoskeletal proteins	Vimentin	+++	+	++
	Cytokeratin 5/6	+++	++	+
	Desmin	-	-	-
Cell adhesion molecules	Connexin43	+++	++	++
	E-cadherin	+	++	++
Steroidogenic hormones	StAR	+++	+	++
	P450scc	++	-	-
	3B-HSD	++	+	+
Steroid hormone receptors	ER-α	+	+++	+++
	ER-β	++	+++	+++
	PR-α	++	-	-
	PR-β	+	++	++
Gonadotropin receptors	LH receptor	+++	+	+
	FSH receptor	++	+	+
SV40 Large T antigen		-	+	+
AIG (Anchorage-independent growth)		-	-	-
Extended growth potential		-	+	+
Progesterone production		**N/A**	+	+

Positive cells: -, none ; +, Weekly positive ; ++, Relative positive ; Stongly positive. N/A, not available.

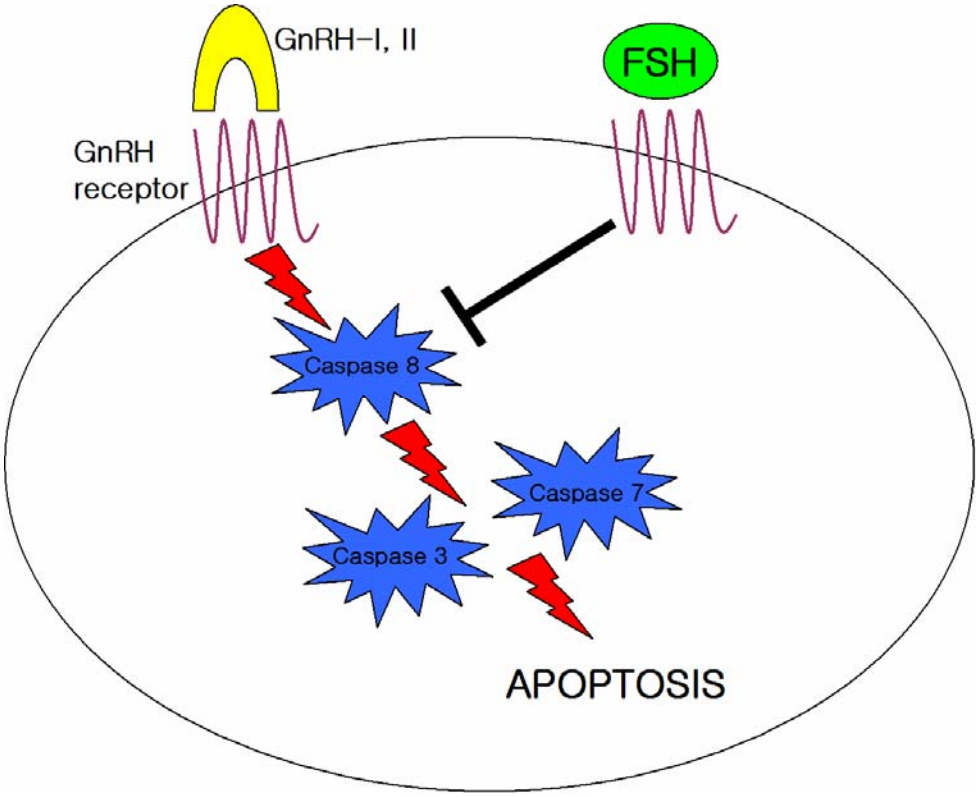

Figure 38. Molecular mechanisms of GnRH-I or II induced apoptosis in human granulosa cells. The direct apoptosis-inducing effects of GnRH-I or II were mediated by specific GnRH-I receptor. Both GnRH-I and II induced activation of initiator caspase-8 and then, activated initiator caspase-8 directly cleaved effector caspases-3 and -7. GnRH-I or II induced activations of caspases 8, 3 and 7 were attenuated by FSH.

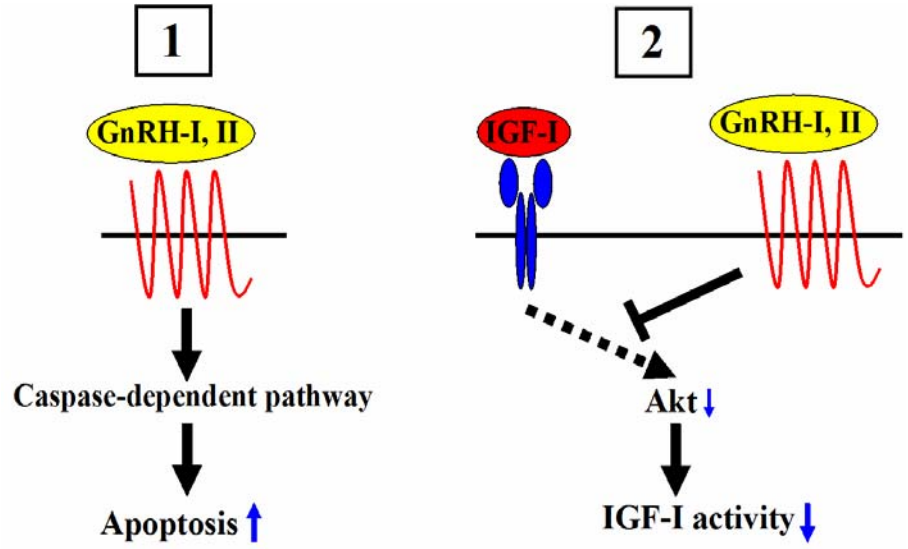

Figure 39. Molecular mechanisms of GnRH-I or II induced anti-proliferative effect in human granulosa cells. The apoptotic effects of GnRH-I or II might be mediated through caspase-dependent extrinsic pathway, rather than Bcl-2 family dependent intrinsic pathway in human granulosa cells. We also have demonstrated that the inhibitory effects of GnRH-I or II on IGF-I activities might be mediated through Akt signaling pathway, rather than the IGF-I receptor phosphorylation and expression in human granulosa cells.

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