INFLUENCES OF ENDOCRINE AND AUTOCRINE FACTORS IN NORMAL AND NEOPLASTIC OVARIAN SURFACE EPITHELIUM

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ABSTRACT

The common epithelial ovarian tumors appear to arise from the ovarian surface epithelium (OSE), which is a simple squamous-to-cuboidal mesothelium covering the ovary. The exact mechanism of ovarian tumorigenesis is not well known even though this disease is the most frequent cause of cancer death in gynecological malignancies. Repeated ovulation contributes to neoplastic transformation of OSE, indicating that the process of healing ruptured OSE may contribute to the disease. Therefore, it has been hypothesized that endocrine and autocrine factors may influence the occurrence of ovarian tumors in women. Recently, non-tumorigenic and tumorigenic immortalized OSE (IOSE) cells were generated by sequentially introducing simian virus 40 (SV40)-large T antigen (IOSE-29) and E-cadherin (IOSE-29EC) into normal OSE. These IOSE-29EC cells were found to be anchorage independent and formed transplantable, invasive subcutaneous and intraperitoneal adenocarcinomas in SCID mice. Thus, two additional cell lines, designated IOSE-29EC/T4 and IOSE-29EC/T5 were established from tumors that arose in IOSE-29EC-inoculated SCID mice. This experimental culture model provides a unique system to examine the influences of endocrine and autocrine factors in OSE at progressive stages of neoplastic transformation.

In the present study, the effects of activin, transforming growth factor (TGF)-β, estradiol (E2), follicle-stimulating hormone (FSH) and gonadotropin-releasing hormone-II (GnRH-II) were investigated in the growth-stimulation or -inhibition and regulation of apoptosis in normal and neoplastic OSE cells. Different levels of activin/inhibin and activin receptor isoforms were expressed in normal and neoplastic OSE cells. In addition, the altered expression of the activin/inhibin subunits, as well as the cell proliferative effect of activin observed in OVCAR-3 but not in normal OSE cells, indicate that activin may act as an autocrine regulator of neoplastic OSE progression. Interestingly, activin and TGF-β inhibited growth and induced apoptosis in early neoplastic (IOSE-29) and tumorigenic OSE (IOSE-29EC) cells. Furthermore, the anti-apoptotic bcl-2 protein was down-regulated by TGF-β, whereas no difference was observed in bax protein by activin or TGF-β treatment and in bcl-2 protein by activin. These results suggest that activin and TGF-β may play a role in growth inhibition and induction of apoptosis in early neoplastic and tumorigenic stages of ovarian cancer. In terms of regulation of apoptosis by E2, it has been demonstrated that IOSE cell lines expressed both ERα and ERβ at the mRNA and

protein levels. In addition, treatment with E2 prevented tamoxifen induced-apoptosis through ERs. The mechanism of E2 action may be associated with up-regulation of bcl-2 gene at the mRNA and protein levels in IOSE-29EC cells. These results suggest that estrogen may play a role in ovarian tumorigenesis by preventing apoptosis in tumorigenic OSE cells. In addition, FSH receptor (FSH-R) was expressed and FSH induced a growth-stimulation in normal and neoplastic OSE cells. Interestingly, FSH stimulated the activation of the MAPK cascade and activated MAPK phosphorylated Elk-1 in neoplastic OSE cells. These results suggest that the MAPK cascade may be involved in cellular function such as growth stimulation in response to FSH in neoplastic OSE cells. GnRH-II mRNA is expressed in normal OSE, immortalized OSE (IOSE), ovarian tumors from the patients and ovarian cancer cell lines, suggesting that GnRH-II exerts an autocrine/paracrine effect in these cells. Treatments with increasing doses (10^{-9} – 10^{-7} M) of GnRH-I and –II resulted in growth-inhibition and induction of apoptosis in IOSE-29 and IOSE-29EC cells. These results suggest that GnRH-II may be an integral regulator similar to GnRH-I in normal OSE physiology and may play a role in the induction of a growth-inhibitory response in neoplastic OSE cells. Taken together, these results suggest that these endocrine and autocrine factors may play a role in ovarian tumorigenesis in the regulation of growth-stimulation or –inhibition and/or apoptosis of normal and neoplastic OSE cells *via* their specific receptors.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
AR	Androgen receptor
ATP	Adenosine 5'-triphosphate
BFGF	Basic fibroblast growth factor
BH1-4	Bcl-2 homology regions 1-4
bp	Base pairs
C	Celcius
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
c-fms	Receptor for colony stimulating factor-1
cGMP	Cyclic guanosine monophosphate
Ci	Curie
Cpm	Counts per minute
DDT	Dithiothreitol
DEPC	Diethylpyrocarbonate
DHT	5α-dihydrotestosterone
DNTP	Deoxynucleoside triphosphate
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
EDTA	Ethylene diaminetetraacetic acid
E2	17β-estradiol
EGF	Epidermal growth factor
EGF-R	EGF receptor
ELISA	Enzyme-linked immunosorbant assay
ER	Endoplasmic reticulum
ER α/β	Estrogen receptor α/β
ERE	Estrogen response element
ERK1/2	Extracellular signal-regulated kinase 1/2
Fas L	Fas ligand
FBS	Fetal bovine serum
FSH	Follicle-stimulating hormone
FSH-R	FSH receptor
G	Acceleration of gravity
GDP	Guanosine diphosphate
GnRH	Gonadotropin-releasing hormone
GnRHa	Gonadotropin-releasing hormone agonist
GnRH-II	Gonadotropin-releasing hormone-II
GnRH-R	Gonadotropin-releasing hormone receptor
G-protein	GTP-binding protein
GPCR	G-protein coupled receptors
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony stimulating factor

GTP	Guanosine triphosphate
H	Hour
HBSS	Hank's balanced salt solution
HCG	Human chorionic gonadotropin
HGF	Hepatocyte growth factor
HGF-R	HGF receptor
HGLCs	Human granulosa-luteal cells
HMG	Human menopausal gonadotropin
IC	Inclusion cyst
ICE	Interleukin-1β (IL-1β) converting enzyme
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor binding protein
IGF-R	IGF receptor
IL	Interleukin
IOSE	Immortalized ovarian surface epithelium
IP	Inositol phosphate
IP_3	Inositol 1, 4, 5-triphosphate
IU	International unit
JNK/SAPK	c-jun terminal kinase/stress-activated protein kinases
Kb	Kilobase
KDa	Kilodaltons
LH	Luteinizing hormone
LH-R	LH receptor
LMP	Low malignant potential
LPA	Lysophosphatidic acid
μ	Micro
mAb	Monoclonal antibody
M-CSF	Macrophage colony-stimulating factor
MAPK	Mitogen-activated protein kinase
MAPKKs (=MKK)	MAPK kinases
MAPKKKs	MAPKK kinases
MAPKKKKs	MAPKKK kinases
MEK1/2	MAPK/ERK kinase 1/2
Ml	Mililiters
Min	Minutes
MMP	Matrix metalloproteinases
MRNA	Messenger ribonucleic acid
Mw	Molecular weight
n (as in nM)	Nano
OCAF	Ovarian cancer activating factor
OSE	Ovarian surface epithelium
p (as in pM)	Pico
P4	Progesterone
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphatase buffered saline

PCD	Programmed cell death
PCR	Polymerase chain reaction
PD-ECGF	Platelet-derived endothelial cell growth factor
PDGF	Platelet-derived growth factor
PDGF-R	PDGF receptor
PGF2α	Prostaglandin F2α
PI	Phosphatidylinositol
PIP	Phosphatidylinositol 4-phosphate
PIP_2	Phosphatidylinositol 4, 5-phosphate
PIP_3	Phosphatidylinositol 3,4,5-triphosphate
PI3K	Phosphatidyl inositol 3 kinase
PKA	Protein kinase A
PKC	Protein kinase C
PLA	Phospholipase A
PLC	Phospholipase C
PLD	Phospholipase D
PMA	Phorbol 12-myristate 13-acetate
PMSF	Phenylmethylsulfonyl fluoride
PR	Progesterone receptor
RIA	Radioimmunoassay
rpm	Revoultions per min
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
SCID	Severe combined immunodeficient
sec	Seconds
SD	Standard deviation
SDS	Sodium dodecyl sulphate
Taq	*Thermus aquaticus*, source of a DNA polymerase
TCF	Ternary complex factor
TE	Tris-EDTA
TEMED	N, N, N', N'-tetramethylethlenediamine
TGF-α	Transforming growth factor-α
TGF-β	Transforming growth factor-β
TβRII	TGF-β receptor
TIMP	Tissue inhibitor of metalloproteinase
TM	Transmembrane
TNF-α	Tumor necrosis factor-α
Tris	Tris(hydroxy methyl) aminomethane
Txf	Tamoxifen
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
VPF	Vascular permeability factor/
v/v	Volume per volume
w/v	Weight per volume

LIST OF PUBLICATIONS

PEER-REVIEWED PAPERS

1. **Choi K-C,** Kang SK, Nathwani PS, Cheng KW, Auersperg N, Leung PCK 2001 Differential expression of activin/inhibin subunit and activin receptor mRNAs in normal and neoplastic ovarian surface epithelium (OSE). *Mol Cell Endocrinol* 174:99-110

2. **Choi K-C**, Kang SK, Tai C-J, Auersperg N, Leung PCK 2001 The regulation of apoptosis by activin and TGF-β in early neoplastic and tumorigenic ovarian surface epithelium (OSE). *J Clin Endcrinol Metab* 86:2125-2135

3. **Choi K-C**, Kang SK, Tai C-J, Auersperg N, Leung PCK 2001 Estradiol up-regulates anti-apoptotic bcl-2 mRNA and protein in tumorigenic ovarian surface epithelium (OSE). *Endocrinology* 142:2351-2360

4. **Choi K-C**, Kang SK, Tai C-J, Auersperg N, Leung PCK 2001 Follicle stimulating hormone (FSH) activates mitogen-activated protein kinases (MAPKs) in neoplastic ovarian surface epithelium (OSE) cells. *J Clin Endcrinol Metab* Submitted

5. **Choi K-C**, Auersperg N, Leung PCK 2001 Expression and role of second form of gonadotropin-releasing hormone (GnRH) in normal and neoplastic ovarian surface epithelium (OSE) cells. *J Clin Endcrinol Metab* In press

6. **Choi K-C**, Tai C-J, Auersperg N, Leung PCK 2001 Adenosine triphosphate (ATP) activates mitogen-activated protein kinases (MAPKs) in neoplastic ovarian surface epithelium (OSE) cells. *Endocrinology* Submitted

7. Kang SK, **Choi K-C**, Cheng KW, Nathwani PS, Auersperg N, Leung PCK 2000 Role of gonadotropin-releasing hormone as an autocrine growth factor in human ovarian surface epithelium. *Endocrinology* 141:72-80

8. Kang SK, **Choi K-C**, Tai C-J, Auersperg N, Leung PCK 2001 Estradiol regulates gonadotropin-releasing hormone (GnRH) and its receptor gene expression and modulates the growth inhibitory effects of GnRH in human ovarian surface epithelial and ovarian cancer cells. *Endocrinology* 142:580-588

9. Tai C-J, **Choi K-C**, Fluker M, Leung PCK 2001 Extracellular adenosine triphosphate induces apoptosis in human granulosa-luteal cells. *J Clin Endcrinol Metab* Submitted

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11. Tai C-J, Kang SK, **Choi K-C**, Leung PCK 2001 Prostaglandin F2α activates mitogen-activated protein kinase in human granulosa-luteal cells. *J Clin Endcrinol Metab* 86:375-380

12. Tai C-J, Kang SK, **Choi K-C**, Tzeng C-R, Leung PCK 2001 Antigonadotropic action of ATP in human granulosa-luteal cells: Involvement of PKCα. *J Clin Endcrinol Metab* In press

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14. Nathwani PS, Kang SK, Cheng KW, **Choi K-C**, Leung PCK 2000 Regulation of gonadotropin-releasing hormone (GnRH) and its receptor gene expression by 17β-estradiol in cultured human granulosa-luteal cells. *Endocrinology* 141:1754-1763

15. Tai C-J, Kang SK, Cheng KW, **Choi K-C**, Nathwani PS, Leung PCK 2000 Expression and regulation of purinergic receptor (P2UR) in human granulosa-luteal cells. *J Clin Endo Metab* 85:1591-1597

16. Kang SK, Tai C-J, Nathwani PS, **Choi K-C**, Leung PCK 2001 Stimulation of mitogen-activated protein kinase by gonadotropin-releasing hormone in human granulosa-luteal cells. *Endocrinology* 142:671-679

17. Kang SK, Cheng KW, Ngan ESW, Chow BKC, **Choi K-C**, Leung PCK 2000 Differential expression of human gonadotropin-releasing hormone receptor gene in pituitary and ovarian cells. *Mol Cell Endocrinol* 162:157-166

ABSTRACTS AND ORAL PRESENTATIONS

1. **Choi K-C,** Kang SK, Nathwani PS, Cheng KW, Tai C-J, Auersperg N, Leung PCK (Poster Presentation) The expression levels of activin/inhibin subunits and activin receptors: Autocrine function of activin in ovarian surface epithelium and ovarian cancer. *1999 Health Sciences Student Research Forum Poster Presentation,* Woodward Instructional Resources Center, University of British Columbia, Vancouver, BC, Canada (Oct 13, 1999)

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3. **Choi K-C,** Tai C-J, Auersperg N, Leung PCK (Poster Presentation) Estradiol up-regulates anti-apoptotic bcl-2 mRNA and protein in tumorigenic ovarian surface (OSE) cells. *UBC/C&W Student Research Forum Poster Presentation*, Children's & Women's Health Center of British Columbia, Poster #13 (March 5, 2001)

4. Tai C-J, Kang SK, **Choi K-C**, Leung PCK (Oral Presentation) Prostaglandin F2α activates mitogen-activated protein kinase in human granulosa-luteal cells. *Fertil Steril* 74 (3S): S2-S3

5. **Choi K-C**, Auersperg N, Leung PCK (Oral Presentation) Estradiol up-regulates anti-apoptotic bcl-2 mRNA and protein in tumorigenic ovarian surface (OSE) cells. *UBC/Dept of OB & GYN Research Day Presentation*, Chan Auditorium, BC Women's Hospital (April 19, 2001)

6. Tai C-J, Kang SK, **Choi K-C**, Leung PCK (Poster Presentation) Role of mitogen-activated protein kinase in prostaglandin F2α action in human granulosa-luteal cells. *UBC/C&W Student Research Forum Poster Presentation*, Children's & Women's Health Center of British Columbia, Poster #47 (March 5, 2001)

7. Kang SK, Cheng KW, **Choi K-C**, Leung PCK (Poster Presentation) Combinations of cell-specific factors may explain the differential expression of human gonadotropin-releasing hormone receptor gene in pituitary and ovarian cells. *UBC/Dept of OB & GYN Research Day Presentation*, Chan Auditorium, BC Women's Hospital (April 19, 2001)

8. Tai C-J, Cheng KW, Nathwani PS, **Choi K-C**, Leung PCK (Poster Presentation) The role of protein kinase C in regulating ATP-evoked intracellular calcium oscillations and enhancing progesterone production in cultured human granulosa-luteal cells. *Biology of Reproduction.* 60(Supp 1): 131, 1999

9. Tai C-J, Kang SK, Cheng KW, **Choi K-C**, Nathwani PS, Leung PCK (Oral Presentation) Expression of purinergic receptor (P2UR) in human granulosa-luteal cells. *ASRM/CFAS '99 Annual Meeting Program Supplement* S-47, 1999

AWARDS

1. Excellent Student Scholarship of Seoul National University
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I. BACKGROUND

1. Ovarian surface epithelium

1.1. Prologue

The ovarian surface epithelium (OSE), also referred to in the literature as ovarian mesothelium (OM) (Nicosia *et al.*, 1991; 1997), is the modified pelvic mesothelium that covers the ovary. It is composed of a single layer of flat-to-cuboidal epithelial cells with few distinguishing features (Nicosia *et al.*, 1991). The OSE used to be referred to as the 'germinal epithelium' as it was once mistakenly believed that it could give rise to new germ cells. Since this hypothesis was disclaimed, ovarian research has centered on the components of the ovary that carry out its important and highly complex endocrine and reproductive functions. Interest in the OSE revived when it became apparent that approximately 90% of human ovarian cancers, epithelial ovarian carcinomas, might arise in the OSE (Auersperg *et al.*, 1998; Herbst, 1994 Nicosia *et al.*, 1991). The implication of OSE as the source of epithelial ovarian cancers was questioned (Dubeau, 1999) because it was based mainly on histopathologic and immunocytochemical observations. Animal models were not available because, except in aging hens (Fredrickson, 1987), ovarian tumors in species other than human do not arise in OSE but in follicular, stromal or germ cells, and the biology of these tumors is fundamentally different from that of epithelial ovarian cancer. Because of the resulting lack of experimental models, the etiology and early events in ovarian carcinogenesis are still among the least understood of all major human malignancies. The first tissue culture systems for OSE from human (Auersperg *et al.*, 1984; Siemens and Auersperg, 1988) were developed. Subsequently, information about the normal functions of OSE and its relationship to ovarian cancer expanded rapidly and, recently,

the capacity of cultured OSE to give rise to ovarian adenocarcinomas was demonstrated experimentally (Auersperg *et al.*, 1999; Ong *et al.*, 2000). The results of these studies indicate that OSE is physiologically much more complex than would be predicted from its inconspicuous appearance, and they support the hypothesis that the ovarian epithelial cancers arise in this simple epithelium.

1.2. Structure

In the mature woman, OSE is an inconspicuous monolayered squamous-to-cuboidal epithelium (Fig. 1). It is characterized by the keratin types 7, 8, 18 and 19 which represent the keratin complement typical for simple epithelia. It expresses mucin antigen MUC1, 17β-hydroxysteroid dehydrogenase and cilia, which distinguish it from extraovarian mesothelium, apical microvilli and basal lamina (Auersperg *et al.*, 1994; Blaustein and Lee 1979; Siemens and Auersperg 1988). Intercellular contact and epithelial integrity of OSE are maintained by simple desmosomes, incomplete tight junctions (Siemens and Auersperg 1988), several integrins (Cruet *et al.*, 1999; Kruk *et al.*, 1994) and cadherins (Davies *et al.*, 1998; Sundfeldt *et al.*, 1997).

The OSE is separated from the ovarian stroma by a basement membrane and, underneath, by a dense collagenous connective tissue layer, the tunica albuginea, which is responsible for the whitish color of the ovary. It is thinner and less resilient than the tunica albuginea in the testis, but likely provides a partial barrier to the diffusion of bioactive agents between the ovarian stroma and the OSE. The OSE differs from all other epithelia by its tenuous attachment to its basement membrane, from which it is easily detached by mechanical means. Until recently, the resulting loss of OSE in surgical specimens was responsible for the widely held opinion that OSE is frequently absent in ovaries of older women.

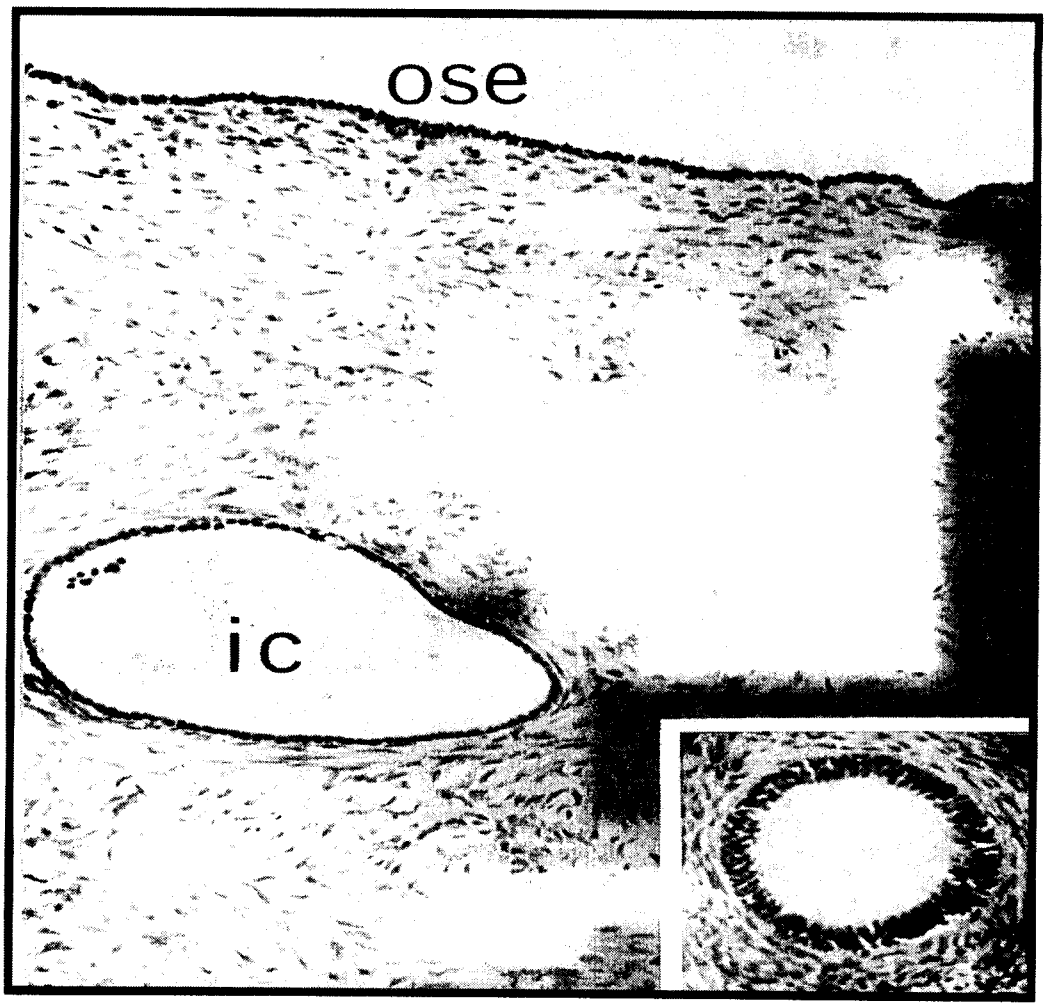


Figure 1. Morphology of normal OSE and inclusion cyst (ic). Section through a normal adult ovarian cortex, showing OSE on top as a cuboidal monolayer and an epithelial inclusion cyst lined with OSE. The *inset* illustrates an inclusion cyst that has undergone tubal metaplastic changes as indicated by the densely arranged, columnar epithelial cells. Hematoxylin & eosin, X 80 (Auersperg *et al.*, 2001).

Whether this loose attachment has any physiological consequences is not known. With age, the human ovary assumes increasingly irregular contours and forms OSE-lined surface invaginations (clefts) and epithelial inclusion cysts in the ovarian cortex. It has been suggested that the squamous and cuboidal forms of OSE cells on the ovarian surface represent cell groups that, respectively, have or have not undergone postovulatory proliferation (Gillett *et al.*, 1991). In addition, OSE cells tend to assume columnar shapes, especially within clefts and inclusion cysts. Whether these shape changes are the result or crowding of whether they reflect genetically determined metaplastic changes is not always clear, but they may be derived by either process.

The importance of surface invaginations and inclusion cysts lies in the propensity of the OSE in these regions to undergo metaplastic changes, i.e. to take on phenotypic characteristics of Mullerian (usually tubal) epithelium, which include columnar cell shapes and several markers found in ovarian neoplasms, including CA125 and E-cadherin (Maines-Bandiera and Auersperg 1997; Mittal *et al.*, 1995; van Niekerk *et al.*, 1991; Sundfeldt *et al.*, 1997) Furthermore, OSE-lined clefts and inclusion cysts, rather than surface OSE, are not only common sites of benign metaplasia but also of early neoplastic progression (Deligdisch *et al.*, 1995; Scully, 1995a; 1995b). It has been suggested that the inclusion cysts form from OSE fragments that are trapped in or near ruptured follicles at the time of ovulation (Murdoch, 1994). However, inclusion cysts have been reported to be more numerous in ovaries of multiparous women than in nulliparous women who ovulate more frequently, and the cysts are particularly numerous in women with polycystic ovarian disease, a condition which is characterized by anovulation or infrequent ovulation (Scully, 1995b), proposing as an alternative that inclusion cysts arise through inflammatory adhesions of surface OSE which becomes apposed at sites of surface invaginations, combined with localized stromal proliferation.

There is currently no definitive explanation for the predilection of inclusion cysts as preferred sites of neoplastic progression of OSE but these preferential locations strongly suggest the presence of specific tumor-promoting microenvironmental factors in these sites. Two different scenarios can be envisaged: *(1)* OSE within inclusion cysts is not separated from underlying stroma by the tunica albuginea. Therefore, this OSE likely has more access to stromally derived growth factors and cytokines as well as to blood-born bioactive agents which may promote neoplastic progression. This hypothesis is supported by the observation that, in inclusion cysts located near the ovarian surface, metaplastic and dysplastic changes tend to be more pronounced on the side near the stroma than on the side adjacent to the tunica albuginea (Scully, 1995a; 1995b). *(2)* Neoplastic progression in OSE-lined cysts and clefts may be promoted by autocrine mechanisms through OSE-derived cytokines and hormones, since these agents may accumulate to bioactive levels in such confined sites but not on the ovarian surface where they diffuse into the pelvic cavity. The hypothesis that these factors participate in autocrine loops is supported by the capacity of normal OSE to secrete bioactive cytokines including interleukin (IL)-1 and IL-6 (Ziltener *et al.*, 1993) and by reports that IL-1 and IL-6 enhance the proliferation of ovarian carcinomas (Berchuck *et al.*, 1993), and that IL-1 causes changes in gene expression including the induction of tumor necrosis factor (TNF)-α which is a mitogen for OSE (Wu *et al.*, 1992; 1993). Within inclusion cysts, such cytokines and hormones might act as immediate autocrine growth regulators, or they might cause secondary changes in gene expression which promote neoplasia.

1.3. Functions

The OSE transports materials to and from the peritoneal cavity and takes part in the cyclical ovulatory ruptures and repair. Most of these functions vary with the reproductive cycle and thus

are likely hormone dependent (Nicosia *et al.*, 1991; Osterholzer *et al.*, 1985). There have been several reports based on electron microscopy and histochemistry, suggesting that the OSE contains lysosome-like inclusions and produces proteolytic enzymes which may contribute to follicular rupture (Bjersing and Cajander, 1975). These reports were supported by direct observations of protease secretion by cultured OSE (Kruk *et al.*, 1994). However, this concept has been questioned because of inconsistencies in the timing of the appearance of the dense lysosome-like granules in the OSE, their biochemical nature, and the observation that follicles denuded of overlying OSE can also rupture (Espey and Lipner, 1994). Furthermore, electron microscopy in various species has revealed that OSE cells degenerate and slough off the follicular surface shortly prior to ovulatory rupture. There is evidence that this cyclic, localized loss of OSE near the time of ovulation is due to apoptosis which is induced by prostaglandins (Ackerman and Murdoch, 1993; Murdoch, 1995) and perhaps mediated by the Fas antigen (Baldwin *et al.*, 1999; Quirk *et al.*, 1997). It is possible that, as the tunica albuginea in the area of the stigma thins and ultimately disappears prior to ovulation, the OSE in this region is exposed to stromal influences which induce apoptosis. However, the possibility cannot be ruled out that the OSE alters the tunica albuginea and underlying stroma in the area of incipient ovulation just prior to its disappearance. The proteolytic capacity of OSE might contribute to the remodeling of the ovarian cortex, as well as the breakdown. OSE likely also takes part in the restoration of the ovarian cortex by the synthesis of both epithelial and connective tissue-type components of the extracellular matrix (Auersperg *et al.*, 1994; Kruk *et al.*, 1994; Kruk and Auersperg, 1994) and by its contractile activity which resembles the contractile capacity exhibited by connective tissue fibroblasts during wound healing (Kruk and Auersperg, 1992). Like fibroblasts, which convert to myofibroblasts when engaged in tissue repair, OSE cells in culture contain smooth muscle actin.

This is in keeping with their dual epithelio-mesenchymal phenotype, and with the proposition that OSE cells, like many other cell types, acquire a regenerative rather than stationary phenotype when they are explanted into culture. Contraction by OSE cells may also play a role in the shrinkage of the ovaries that occurs with age and results in their typical convoluted shape and the formation of the OSE-lined clefts and inclusion cysts.

1.4. Differentiation

Normal OSE covering a non-ovulating ovary is a stationary mesothelium with both epithelial and mesenchymal characteristics. In contrast to mesothelia, OSE retains the capacity to alter its state of differentiation along pathways leading either to stromal, or to epithelial phenotypes. In response to stimuli that initiate a regenerative (repair) response, such as ovulatory rupture *in vivo* or explantation into culture, OSE cells assume phenotypic characteristics of stromal cells. Alternatively, OSE acquires complex epithelial characteristics of the Mullerian duct derived epithelia, i.e. of the oviduct, endometrium and endocervix, when it undergoes metaplasia, benign tumor formation, and neoplastic progression. Together, these characteristics show that the differentiation of OSE is not as firmly determined as in other adult epithelia and that OSE is closer to its pleuripotential mesodermal embryonic precursor form than other coelomic epithelial derivatives.

Normal stationary OSE has no known tissue-specific differentiation markers. *In situ*, it can be distinguished from extraovarian mesothelium by the lack of CA125 and by the differential expression of mucin, cilia, 17β-hydroxysteroid dehydrogenase and several antigenic markers (Auersperg *et al.*, 1998; van Niekerk *et al.*, 1989; 1991; Zeimet *et al.*, 1998). It has classical epithelial features which include desmosomes, tight junctions, basement membrane, keratin and

apical microvilli, but other aspects of epithelial differentiation are less defined. For example, E-cadherin and CA125 in human OSE are rare while both markers occur in oviductal and endometrial epithelium, and CA125 is also secreted by extraovarian pelvic mesothelium and by abdominal and pleural peritoneum (Nicosia *et al.*, 1991; van Niekerk *et al.*, 1989; Zeimet *et al.*, 1998). OSE cells also constitutively coexpress keratin with vimentin which is a mesenchymal intermediate filament, expressed by most epithelial cells only in response to wounding, explantation into culture or pathological conditions (Gilles *et al.*, 1999; Hornby *et al.*, 1992). Expression of the connective tissue collagen types I and III has been shown in cultured OSE, but not *in situ* (Auersperg *et al.*, 1994).

During postovulatory repair and in culture, OSE cells have the ability to modulate to a fibroblast-like form which reflects their close developmental relationship to ovarian stromal cells. The exact mechanisms regulating this conversion have not been defined. However, epidermal growth factor (EGF), collagen substrata and ascorbate are all conducive to epithelio-mesenchymal conversion of OSE in culture. In addition, transforming growth factor (TGF)-β, which is an autocrine regulator of OSE growth (Berchuck *et al.*, 1992), causes epithelio-mesenchymal conversion in a number of epithelial cell types (Toda *et al.*, 1997). Similar epithelio-mesenchymal conversions occur *in vivo* in mesodermally derived cell types closely related to OSE, such as pleural mesothelial cells responding to injury (Davila and Crouch, 1993). This capacity of OSE to undergo epithelio-mesenchymal conversion likely confers advantages during the post-ovulatory repair of the ovarian surface: it increases motility, alters proliferative responses and capacities to modify extracellular matrix, and renders the cells contractile. Epithelio-mesenchymal conversion might also function as a homeostatic mechanism to accommodate OSE cells that become trapped within the ovary at ovulation, to allow them to

become incorporated into the ovarian stroma as stromal fibroblasts. As a related hypothesis, an inability to undergo epithelio-mesenchymal conversion would preserve the epithelial forms within the ovarian stroma which could lead to OSE cell aggregation and subsequent inclusion cyst formation (Fig. 2). In contrast to epithelio-mesenchymal conversion which is part of normal OSE physiology, the differentiation of metaplastic and neoplastic OSE along the lines of Mullerian duct-derived epithelia is clearly a pathological process, based on complex epigenetic and genetic changes.

1.5. OSE in culture

1.5.1. Culture methods

The detailed procedures used for isolating and culturing normal human OSE were summarized previously (Kruk *et al.*, 1990). Briefly, specimens for culture are obtained from overtly normal ovaries at surgery for nonmalignant gynecological diseases. Fragments of OSE are gently scraped from the ovarian surface with a rubber scraper or with the blunt side of a scalpel or other suitable instrument and immediately placed into sterile culture medium, taking care that the tissue remain sterile and does not dry, which happens very rapidly. OSE is also very loosely attached to the underlying ovarian cortex and is easily lost by excessive handling. If the surgery involves the removal of the ovaries, the OSE is obtained either by the surgeon while the ovaries are still *in situ*, or by a member of the research team after removal from the patient. OSE can also be obtained by the surgeon laparoscopically at the time of minor gynecologic procedures which are carried out by this approach. The OSE fragments are cultured in medium 199:MCDB 105 (1:1) with 15% fetal bovine serum (FBS). In addition, either 50 μg/ml gentamicin or 100 μg/ml of penicillin/streptomycin is added for the first few weeks.

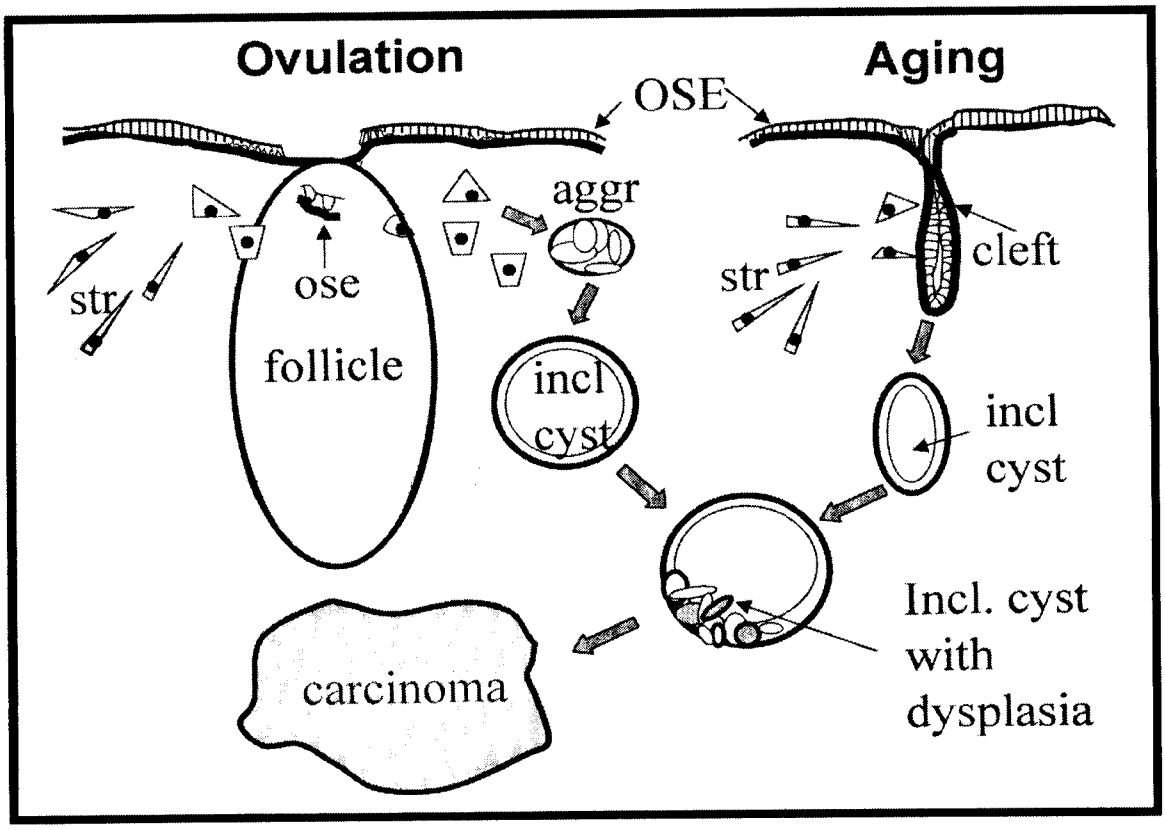


Figure 2. Epithelio-mesenchymal conversion of OSE cells may represent a homeostatic mechanism to incorporate cells that have been displaced from the ovarian surface into the stroma. If such conversion does not take place, the cells are more likely to form epithelial inclusion cysts which are preferred sites of neoplastic progression. Diagram outlining two paths by which OSE is displaced into the ovarian cortex. OSE fragments are displaced into or near the ruptured follicle at ovulation. OSE also lines surface invaginations, or clefts, which form as the ovary ages. If OSE cells undergo epithelio-mesenchymal conversion, they may migrate into, and become part of the stroma *(str)*. Alternatively, the cells remain epithelial, aggregate *(aggr)* and form inclusion cysts ***(incl cyst).*** Cysts may also form through the pinching off of surface clefts. Inclusion cysts are preferred sites of metaplastic and dysplastic changes which may lead to tumorigenesis. Importantly, the capacity of OSE to undergo epithelio-mesenchymal conversion is greatly reduced with malignant progression and, to a lesser degree, in women with a genetic predisposition to develop ovarian cancer (Auersperg *et al.*, 2001).

The cultures are left undisturbed for at least 4 days, grown to confluence and then routinely passaged and split 1:3 when confluent, with 0.06% trypsin (1:250) and 0.01% EDTA. The cultures usually proliferate for 3-4 passages (1:3 splits) and then senesce. They are defined as senescent if they are composed of large flat cells that do not reach confluence over one month. OSE cells in low passage culture can undergo epithelio-mesenchymal conversion, which tends to extend their life span by a few passages (Fig. 3)(Auersperg *et al.*, 1994). Reduced-serum, and serum-free media were designed for human OSE and used to study mitogenic effects of growth factors and hormones (Elliott and Auersperg, 1993).

Markers to distinguish OSE from cell contaminants in culture include keratins 7, 8, 18 and 19 which distinguish OSE from other ovarian cell types (van Niekerk *et al.*, 1991), 17β-OH steroid dehydrogenase and mucin, which distinguish it from extraovarian mesothelial cells, laminin, which together with keratin distinguishes OSE from stromal fibroblasts; and the absence of factor VIII and Ulex lectin receptors which distinguish OSE from the morphologically similar endothelial cells (Auersperg *et al.*, 1994; Nicosia *et al.*, 1991; Siemens and Auersperg, 1988).

1.5.2. Extension of the life-span of OSE cells

One of the problems in human OSE research is the small number and short lifespan of cells obtained at surgery. To alleviate this problem, "immortalizing" genes such as SV40 large T antigen (Tag) (Maines-Bandiera *et al.*, 1992) and the HPV genes P6 and P7 (Wan *et al.*, 1997) have been introduced into OSE. Expression of these genes does not truly immortalize human

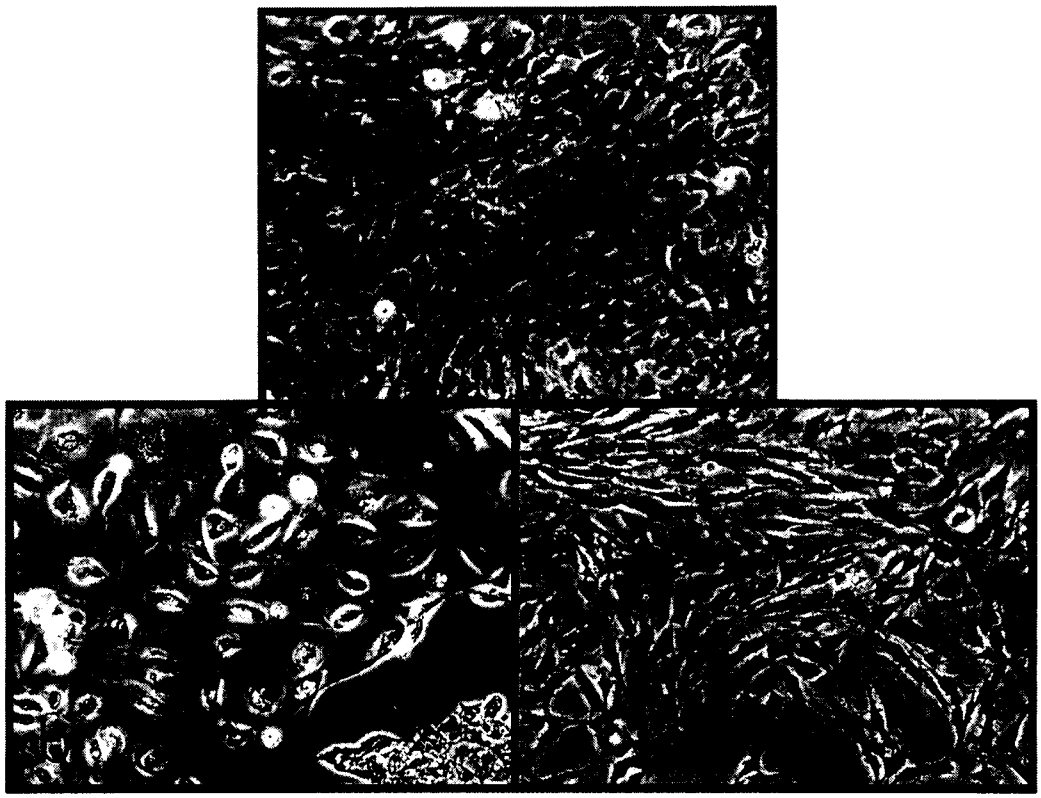


Figure 3. Morphology of OSE in culture. **a**, Primary epithelial culture with a compact, cobblestone-like growth pattern. **b**, Passage 2 with flat epithelial OSE cells. Note a small group of granulosa cells in the lower right corner. **c**, Passage 5 with OSE cells that have undergone epithelio-mesenchymal conversion and have assumed fibroblast-like shapes. Such cells are initially keratin-positive but tend to lose keratin with time and passages in culture (Siemens and Auersperg, 1988). X 200 (Auersperg *et al.*, 2001).

OSE cell lines in that their population doubling capacity is greatly extended but not infinite; however, the lines provide sufficiently large cell numbers for molecular studies. One advantage of these lines is that they tend to retain some, though not all, of the tissue-specific properties of the cells from which they are derived. For example, many of these lines retain keratin, and most, if not all of them, continue to express N-cadherin and lack E-cadherin (in common with normal, and in contrast to neoplastic OSE). Although such lines are nontumorigenic in SCID mice (Ong *et al.*, 2000), their growth controls are profoundly disturbed, which confers on them properties of neoplastic cells such as genetic instability, increased saturation density, reduced serum requirements and variable degrees of anchorage independence. Tag and E6/E7 inactivate the tumor suppressor genes p53 and p105RB (May and May, 1999; Stiegler *et al.*, 1998). Importantly, 30-80% of epithelial ovarian carcinomas have p53 mutations which disrupt controls of the cell cycle, DNA repair and apoptosis (Wen *et al.*, 1999). Sometimes, a few cells of such "immortalized" OSE cultures survive crisis and become truly immortal, continuous lines. Recently, constitutively expressed E-cadherin was introduced into an SV40 Tag–immortalized line derived from normal OSE. The resulting phenotype closely resembled neoplastic OSE, and the cells formed adenocarcinomas in SCID mice (Auersperg *et al.*, 1999; Ong *et al.*, 2000). These adenocarcinomas resembled Mullerian duct-derived epithelia in that they formed papillae and cysts and expressed CA125 and E-cadherin. The line, IOSE-29EC, became not only tumorigenic but also acquired an indefinite, truly immortal growth potential. While the exact relationships between the introduction of T-antigen and E-cadherin to tumorigenicity need to be examined in additional lines, this is the first experimental transformation of normal human OSE to ovarian adenocarcinoma cells (Table 1) and the first direct confirmation that OSE is capable of such a transformation. The results support the hypothesis that E-cadherin may act as an inducer

of the Mullerian epithelial differentiation which accompanies neoplastic conversion of OSE (Wong *et al.*, 1999).

1.6. Regulation by hormones, growth factors and cytokines

Normal OSE cells secrete, and have receptors for agents with growth- and differentiation regulatory capabilities.

1.6.1. Gonadotropin-releasing hormone and gonadotropins

Recently, gonadotropin-releasing hormone (GnRH) has been shown to be an autocrine growth inhibitor for normal OSE. Using RT-PCR and Southern blot analysis, GnRH and the GnRH receptor in human OSE cells were cloned and found to have sequences identical to those found in the hypothalamus and pituitary, respectively (Kang *et al.*, 2000). It has been shown that gonadotropins stimulate cell proliferation of normal OSE of several species *in vivo* and *in vitro* (Davies *et al.*, 1999; Osterholzer *et al.*, 1985). Human OSE cells also have receptors for FSH (Zheng *et al.*, 1996). The presence of these receptors lends support to the hypothesis that the high FSH levels in peri- and postmenopausal women may play a promoting role in ovarian carcinogenesis, since this is the age of the peak incidence of epithelial ovarian carcinomas (te Velde *et al.*, 1998). Human and rabbit OSE express luteinizing hormone (LH) receptors since hCG, which is secreted by human OSE, stimulates their proliferation (Hess *et al.*, 1999) and LH also stimulates rabbit OSE growth in culture (Osterholzer *et al.*, 1985).

1.6.2. Steroids

Steroidogenesis is a very complex process in the ovary (Song and Melner, 2000). Receptors for estrogen, progesterone and androgen were found at the mRNA and/or protein level in human OSE (Karlan *et al.*, 1995; Lau *et al.*, 1999). SV-40 large T-immortalized OSE cells expressed ERα but not ERβ (Brandenberger *et al.*, 1998). No direct effects of these steroids on OSE proliferation have been demonstrated (Karlan *et al.*, 1995), but there is increasing evidence for indirect actions. The expression of GnRH receptor in OSE appears to be reduced by estrogen (Kang *et al.*, 2001) and estrogen also modulates levels of HGF (Liu *et al.*, 1994) and EGF both of which stimulate OSE growth. Furthermore, in ovarian carcinoma cells, estrogen and progesterone markedly influence the steady state levels of mRNA for the HGF receptor Met (Moghul *et al.*, 1994) and 5α-dihydrotestosterone downregulates the expression of mRNA for the TGF-β receptors (Evangelou *et al.*, 2000), suggesting that these steroids may also have indirect effects on the growth regulation of normal OSE. Although there is no evidence for a direct mitogenic effect of ovarian steroids on OSE, it has been known for a long time that corticosteroids enhance OSE proliferation in culture, and that combinations of EGF and hydrocortisone are among the most potent mitogens for cultured OSE (Siemens and Auersperg, 1988). The steroidogenic factor 1 (SF-1), a transcription factor which regulates the differentiation of granulosa cells and inhibits their proliferation, is also growth-inhibitory in rat OSE cells (Nash *et al.*, 1998).

1.6.3. Growth factors

1.6.3.1. Transforming growth factor-β family

Among agents which inhibit OSE growth are several members of the TGF-β family of growth factors (Taipale *et al.*, 1998), which affect and/or are produced by OSE. TGF-β itself, a widely

distributed growth factor with multiple modes of action, acts as an autocrine growth inhibitor for cultured human OSE (Berchuck *et al.*, 1992) and also counteracts the growth-stimulatory effect of EGF (Vigne *et al.*, 1994). In contrast to some other inhibitory factors, TGF-β does not induce apoptosis in OSE cells (Havrilesky *et al.*, 1995). TGF-β inhibits growth of rabbit OSE (Pierro *et al.*, 1996) and regulates Kit ligand expression in immortalized rat OSE (Ismail *et al.*, 1999). A detailed examination by immunohistochemistry and *in situ* hybridization of TGF-β subtypes, the related protein endoglin, TGF-β receptors and TGF-β-binding protein demonstrated the presence of all of these in human OSE and, with the exception of the binding protein, levels were lower than in ovarian cancers (Henriksen *et al.*, 1995). Interestingly, 5α-dihydrotestosterone downregulates the expression of mRNA for the TGF-β receptors I and II in ovarian carcinoma lines (Evangelou *et al.*, 1995), suggesting that it might also counteract growth inhibitory effects of TGF-β in normal OSE. It has been demonstrated that activin, inhibin and follistatin are present in normal and neoplastic ovarian epithelia. OSE, immediately after removal from the ovary, expressed mRNA for follistatin 288 and 315, for the activin receptors IA, IB, II and IIB, as well as for the α subunit and (weakly) the β subunit of the ligands (Welt *et al.*, 1997). At the protein level, OSE produced inhibin only. After 1 month in culture, the α subunit was undetectable while the βA subunit became abundant. Another member of the TGF-β family, anti-Mullerian hormone (AMH), which causes regression of the Mullerian ducts in male fetuses, is produced at low levels by granulosa cells throughout the reproductive life of women (Josso *et al.*, 1998). In view of the close developmental relationship between the Mullerian ducts and OSE, it might be expected that AMH should affect OSE cells.

1.6.3.2. Epidermal growth factor (EGF) family

Among growth factors, those of the EGF family were among the first reported to stimulate human and rabbit OSE proliferation either with or without co-stimulation by corticosteroids (Berchuck *et al.*, 1993; Pierro *et al.*, 1996; Rodriguez *et al.*, 1991; Siemens and Auersperg, 1988). OSE cells express receptors for EGF and TGF-α, which is a structural homologue of EGF and binds to the EGF receptor (Berchuck *et al.*, 1991). EGF not only stimulates proliferation of human OSE cells but also profoundly affects their differentiation: within a few days of EGF treatment, the cells convert from an epithelial to a spindle-shaped morphology and lose epithelial differentiation markers such as keratin (Siemens and Auersperg, 1988). The resulting localized stimulation of the OSE likely contributes to its rapid postovulatory proliferation and perhaps also to epithelio-mesenchymal conversion of OSE cells trapped within the ruptured follicle. TGF-α has been demonstrated immunohistochemically in human OSE *in vivo* and *in vitro*, and found to stimulate thymidine incorporation by cultured human OSE cells. It was also demonstrated immunohistochemically in human theca cells, suggesting that it plays a role in the reproductive functions of the ovary (Jindal *et al.*, 1994). In OSE cells whose lifespan has been extended by transfection with SV40 large T antigen, EGF does not enhance proliferation but promotes survival (McLellan *et al.*, 1999). Amphiregulin, another EGF homologue, is also a potent mitogen for OSE cells and appears to control OSE and ovarian cancer cell proliferation in a complex manner (Gordon *et al.*, 1994; Johnson *et al.*, 1991).

Of particular interest for ovarian cancer are the heregulins, including the heregulin/*neu* differentiation factor, which are a family of ligands that cause phosphorylation of the HER2/neu receptor, a 185 kD transmembrane protein kinase with extensive homology to the EGF receptor (Aguilar *et al.*, 1999). HER1 (synonymous with EGF receptor), HER2, HER3 and HER4 are

members of the type I receptor tyrosine kinase family (RTK I) of epithelial growth factor receptors (Downward *et al.*, 1984). These receptors interact in multiple ways which modify their influence on a variety of cells (Klapper *et al.*, 2000). Though normal OSE cells express EGF receptors, they express little or no HER-2/*neu* (Gordon *et al.*, 1994, Kohler *et al.*, 1992; Owens *et al.*, 1991). However, HER2/neu is amplified and overexpressed in 25-30% of ovarian and breast cancers, and this overexpression is associated with a poor prognosis (Aguilar *et al.*, 1999).

1.6.3. Cytokines

Cultured human OSE also secretes bioactive cytokines, including IL-1, IL-6, macrophage colony-stimulating factor (M-CSF), granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony stimulating factor (GM-CSF). These agents have regulatory effects on follicular growth and differentiation, ovulation, and the distribution of intraovarian cells of the immune system (Ziltener *et al.*, 1993), and IL-1 enhances OSE proliferation (Marth *et al.*, 1996). Little is known about the regulation of cytokine expression in OSE, but it may be relevant that ovarian steroid hormones regulate GM-CSF production by uterine epithelial cells which are developmentally related to OSE (Robertson *et al.*, 1996).

2. Epithelial ovarian carcinomas

2.1. Prologue

The epithelial ovarian carcinomas, i.e. the group derived from the OSE, represent approximately 90% of all human ovarian malignant neoplasms, with the rest originating in granulosa cells or, rarely, in the stroma or germ cells. The poor five year survival (30-40 %) is largely due to the fact that most ovarian carcinomas are inoperable when first discovered and

respond poorly to therapy (Herbst, 1994). The common epithelial ovarian tumors appear to arise from the ovarian surface epithelium (OSE), which is a simple squamous-to-cuboidal mesothelium covering the ovary (Auersperg *et al.*, 1998; Herbst, 1994; Nicosia *et al.*, 1991). This group of tumors is the most lethal among ovarian neoplasms and is the prime cause of death from gynecological malignancies. Because of the resulting lack of experimental models, the etiology and early events in ovarian carcinogenesis are still among the least understood of all major human malignancies. The incessant ovulation theory was suggested, which repeated ovulation contributes to (pre)neoplastic change of OSE, suggesting that wound healing process of ruptured OSE may play a role in the disease in women (Fathalla, 1971). Therefore, endocrine and autocrine factors including hormones and multiple growth factors were suggested to influence the occurrence of ovarian tumors during menstrual cycle (Godwin *et al.*, 1993; Hamilton, 1992; Piver *et al.*, 1991; Rao and Slotman, 1991; Risch, 1998; Shoham, 1994; Westerman *et al.*, 1997).

2.2. Genetic changes

Amplification, altered expression and mutations in a number of oncogenes and tumor suppressor genes play a role in the development of ovarian epithelial neoplasms. Oncogenes which are frequently overexpressed or amplified in ovarian carcinomas include c-myc, in particular in serous adenocarcinomas (Tashiro *et al.*, 1992); K-ras, in particular in mucinous carcinomas which may exhibit enteric mucinous differentiation (Enomoto *et al.*, 1991); and erbB2, EGF-R and *c-fms* (the receptor for colony stimulating factor-1) all of which are associated with a poor prognosis (Berchuck *et al.*, 1990; Kacinski *et al.*, 1989; Kohler *et al.*, 1989). Recently, phosphatidyl inositol 3 kinase (PI3K) and its downstream effector AKT2 were also shown to be amplified in a significant proportion of ovarian carcinomas (Bellacosa *et al.*, 1995;

Shayesteh *et al.*, 1999). Among tumor suppressor genes, p53 is mutated in about 50% of late stage tumors but rarely in low stage tumors and borderline lesions (Berchuck *et al.*, 1994), and the PI3K inhibitor PTEN is mutated in a significant proportion of endometrioid ovarian carcinomas (Obata *et al.*, 1998). Mutations in the tumor suppressor genes BRCA1 and BRCA2 appear to form the basis for most cases of familial ovarian cancer. The expression of a recently described tumor suppressor gene, NOEY2 (ARHI), is decreased specifically in carcinomas of the ovary and breast (Yu *et al.*, 1999).

The epidemiology, histopathology and clinical course of OSE-derived ovarian carcinomas differ profoundly from those of the mesotheliomas which arise in extraovarian mesothelium, e.g. a responsiveness to asbestos exposure, and lack of Mullerian phenotypes. This difference reflects, among other factors, the different developmental histories of these two components of the pelvic peritoneum, while may include inductive signals emanating from the ovary and acting on the developing OSE (Blaustein and Lee, 1979; Nicosia *et al.*, 1997).

2.3. Regulation by hormones, growth factors and cytokines

Ovarian carcinomas also secrete and have receptors for agents with growth-regulatory capabilities. The potential roles of peptide hormones, sex steroids and growth factors in ovarian cancer are described below.

2.3.1. Gonadotropin-releasing hormone (GnRH)

GnRH acts as a key hormone in the regulation of pituitary gonadal axis (Conn, 1994). In addition to its well-documented role in gonadotropin biosynthesis and secretion in the pituitary, an autocrine/paracrine role for GnRH has also been suggested in tumors of the ovary and

endometrium (Emons *et al.*, 1992; 1993a; 1993b; Gallagner *et al.*, 1991). This concept is based on the detection of binding sites for GnRH, as well as the expression of GnRH and its receptor gene transcripts in these tumors. Especially noteworthy is the finding that GnRH and its receptor are expressed in normal and neoplastic OSE cells (Kang *et al.*, 2000). GnRH receptors were detected in about 80% of human ovarian epithelial tumors and in numerous ovarian cancer cell lines such as EFO-21, EFO-27, and OV-1063 (Emons *et al.*, 1997; Miyazaki *et al.*, 1997). *In vivo*, long acting GnRH agonists are thought to act by desensitizing or down-regulating the GnRH receptors in the pituitary, resulting in a subsequent decline in gonadotropins which serve as tumor growth factors. The suppression of endogenous LH and FSH secretion by GnRH-agonist treatment results in growth inhibition of heterotransplanted ovarian cancers in animal models (Peterson *et al.*, 1994). *In vitro,* GnRH and its analogs have been shown to inhibit the growth of a number of GnRH receptor-bearing ovarian cancer cell lines (Emons *et al.*, 1992; 1993a). To improve the therapeutic efficiency of GnRH analogs against cancer cells and to reduce cytotoxicity against normal cells, targeted chemotherapy based on the GnRH receptor has been developed recently (Schally and Nagy, 1999).

The exact mechanism underlying the growth inhibitory effect of GnRH analogs remains to be elucidated. At the ovarian GnRH receptor level, the putative endogenous ligand may stimulate the proliferation of the cells through the receptor, which might be down-regulated by continuous treatment with a potent GnRH agonist. The finding that continuous treatment with GnRH agonists, which is thought to induce receptor down-regulation, inhibited ovarian cancer cell growth, and that this effect was abolished by co-treatment with a specific GnRH antagonist, corroborated this view (Kang *et al.*, 2000; Thomson *et al.*, 1991). Alternatively, the ovarian GnRH receptor might mediate direct antiproliferative effects of GnRH analogs. However, this

notion is not corroborated by the observation that both antagonistic and agonistic analogs have been reported to induce growth inhibition of ovarian cancer cells (Yano *et al.*, 1994). Recently, it has been suggested that the well established GnRH receptor signaling mechanism mediated by phospholipase C (PLC) and protein kinase C (PKC) is likely not involved in the antiproliferative effects of GnRH in tumor cells (Emons *et al.*, 1998). It has been reported that analogues of GnRH reverse the growth stimulatory effect of EGF and insulin-like growth factor (IGF) in cancer cells including carcinomas of the ovary (Emons *et al.*, 1996; Hershkovitz *et al.*, 1993; Marelli *et al.*, 1999), possibly by down regulating their receptor numbers and/or mRNA levels. In addition, it has been demonstrated that GnRH analogs reduce cell proliferation by increasing the portion of cells in the resting phase, G_0-G_1 (Thomson *et al.*, 1991) and inducing cell death or apoptosis (Motomura, 1998; Sridaran *et al.*, 1998). Treatment of ovarian cancer cells with GnRH analogues may induce apoptosis mediated by the Fas ligand-Fas system, which has been shown to trigger apoptosis in a variety of cell types (Nagata and Golstein, 1995). Recently, it has been demonstrated that a GnRH analogue may modulate ovarian cancer cell growth by inhibiting telomerase activity without altering the RNA component of telomerase expression (Ohta *et al.*, 1998).

2.3.2. Gonadotropins

The involvement of gonadotropins in ovarian epithelial cancer development is supported by several observations. A number of epidemiological studies have demonstrated an increased occurrence of ovarian cancer with exposure to high levels of gonadotropins during menopause or infertility therapy (Risch, 1998; Shushan *et al.*, 1996; Whittemore *et al.*, 1992). Clinically, administration of human menopausal gonadotropin (hMG) for ovulation induction may increase

the risk of epithelial ovarian tumors (Shushan *et al.*, 1996). Reduced risk of ovarian cancer is associated with multiple pregnancy, breast feeding and oral contraceptive use which results in lower level and reduced exposure to gonadotropins (Rao and Slotman, 1991; Risch, 1998; Shoham, 1994; Whittemore *et al.*, 1992). Receptors for FSH and LH/CG were demonstrated to be present in normal OSE and ovarian tumors (Konishi *et al.*, 1999; Mandai *et al.*, 1997; Zheng *et al.*, 1996; 2000). As in normal OSE cells, FSH and LH/CG stimulated the growth of some ovarian cancer cells in a dose- and time-dependent manner *in vitro* (Kurbacher *et al.*, 1995; Wimalasena *et al.*, 1992). Elevated levels of gonadotropins may promote the growth of human ovarian carcinoma by induction of tumor angiogenesis *in vivo* (Schiffenbauer *et al.*, 1997). Despite these observations, the roles that elevated levels and prolonged exposure to gonadotropins play in ovarian tumorigenesis remain to be elucidated. For instance, in other reports, increased risk of ovarian cancer development has not been demonstrated in women undergoing ovulation induction for *in vitro* fertilization (Franceschi *et al.*, 1994; Venn *et al.*, 1995). The mechanism by which gonadotropins increase ovarian cancer cell growth is unclear. It has been shown that hCG induced estradiol production in a dose dependent manner, whereas FSH had no such effect in primary cultures of epithelial ovarian cancer cells (Wimalasena *et al.*, 1991). The combined treatment of hCG with estradiol may regulate the growth response of epithelial ovarian cancer cells through the IGF-1 and EGF pathways (Wimalasena *et al.*, 1993). Human CG treatment has been demonstrated to suppress cisplatin-induced apoptosis by 58% in the ovarian carcinoma cell line, OVCAR-3 (Kuroda *et al.*, 1998), suggesting that gonadotropins may play a role in preventing apoptosis. Taken together, gonadotropins may be a contributing factor in ovarian tumorigenesis, presumably by enhancing cell proliferation and/or inhibiting apoptosis.

2.3.3. Sex steroids

Both epidemiological and experimental observations have implicated sex steroids in the pathogenesis and growth regulation of carcinomas arising from the ovary (Chien *et al.*, 1994; Galtier-Dercure *et al.*, 1992; Langon *et al.*, 1994). A number of studies have suggested that the risk of developing ovarian cancer increase with the usage and duration of hormone replacement therapy (Garg *et al.*, 1998; Rodriguez *et al.*, 1995). Estrogens taken as oral contraceptives during premenopausal years are protective, but when used in postmenopausal years as hormone replacement therapy, may increase the risk of ovarian cancer (Clinton and Hua, 1997; Garg *et al.*, 1998; Rao and Slotman, 1991; Risch, 1998; Rodriguez *et al.*, 1995). Breast-feeding, which appears to offer protection, is associated with reduced serum concentrations of estradiol (Liu *et al.*, 1983). In addition to estrogens, other ovarian steroids such as androstenedione, testosterone and progestins have also been implicated as risk factors for ovarian cancer (Rao and Slotman, 1991; Risch, 1998). In patients with ovarian cancer, elevated plasma levels of 17β-estradiol, estrone, progesterone, 20α-hydroxyprogesterone, dehydroepiandrosterone sulfate, androstenedione, and testosterone have been observed and shown to be correlated with tumor volume (Backstrom *et al.*, 1983; Mahlck *et al.*, 1985; 1986a; 1986b; 1988). Elevated levels of sex steroid hormones are thought to be produced by ovarian tumor cells. This notion is supported by the increased levels of sex steroids in the ovarian vein draining the tumor-bearing ovary, as compared with the contralateral ovarian vein and the peripheral blood (Aiman *et al.*, 1986; Heinonen *et al.*, 1986; Kitayama and Nakano, 1990). Exogenous estrogen stimulated the growth of several ER-positive ovarian carcinoma cell lines *in vitro* (Chien *et al.*, 1994; Galtier-Dercure *et al.*, 1992; Langon *et al.*, 1994).

The classical estrogen receptor (ER), now referred to as ERα, and the progesterone receptor (PR) were found in <50% of ovarian tumors, whereas the androgen receptor (AR) was detected in the majority of cases reported (> 80%) (Rao and Slotman, 1991; Risch, 1998). In malignant epithelial ovarian tumors, the concentration of ER is generally higher, while the concentration of PR is generally lower in malignant lesions as compared to that of benign tumors or normal ovaries (Toppila *et al.*, 1986; Vierikko *et al.*, 1983; Willcocks *et al.*, 1983). Also, the presence of a second isoform of estrogen receptor (ERβ) has been reported in normal and malignant ovarian cells in primary cultures or ovarian cancer cell lines (Brandenberger *et al.*, 1998; Lau *et al.*, 1999). Nevertheless, the relationship between receptor content and prognostic factors such as histology, stage and grade is unclear. Several authors found no correlation between estrogen receptor content and histological type or grade of differentiation (Anderl *et al.*, 1988; Harding *et al.*, 1999; Nestok *et al.*, 1988). Others reported that endometrioid tumors express more frequently PR, while serous tumors were more frequently found to be ER positive (Harding *et al.*, 1999; Nestok *et al.*, 1988). Some investigators observed that ER positivity was correlated with poor differentiation (Teufel *et al.*, 1983), whereas others found that well differentiated tumors express ER (Iversen *et al.*, 1986) or both ER and PR more frequently (Creasman *et al.*, 1981). PR status was found to be of significant prognostic value in advanced epithelial ovarian cancer cells (Hempling *et al.*, 1998). However, in other studies, no clinical significance of ER and PR status in epithelial ovarian carcinomas was reported when correlated with age, parity, race, smoking, surgical stage, histologic type, histologic grade, progression-free interval, or patient survival (Masoo *et al.*, 1989). Also, no correlation between the presence of AR and tumor histology was found (Kuhnel *et al.*, 1987; Slotman *et al.*, 1989). The apparent discrepancy of these observations may be explained by differences in the assay methods, the criteria for positivity for steroid

receptors, and/or heterogeneity of tumor cell populations with respect to steroid receptor contents (Slotman *et al.*, 1989). The ERα mRNA mutation with a 32-bp deletion in exon 1 was found in SKOV-3 cell line, which is insensitive to E2 with respect to cell proliferation and induction of gene expression (Lau *et al.*, 1999). This may provide an explanation for the lack of responsiveness and resistance to E2 in some ovarian cancers.

The exact mechanism of action of steroid hormones in ovarian cancer remains unclear. Induction of c-myc oncoprotein has been shown to mediate the mitogenic response to growth stimuli (Chien *et al.*, 1994). Depending on the levels of ER, up-regulation of c-myc protein by estrogen has been shown to mediate estrogen-induced ovarian cancer cell growth. It has been demonstrated that estrogen interacts with other growth factors in the normal ovary and ovarian cancer cells. In the ovarian cancer cell line, PE01, the estrogen-mediated growth stimulatory effects were reversed by an EGF receptor-targeted antibody (Simpson *et al.*, 1998). In addition, estrogen induced a significant increase in TGF-α protein concentration in media and estrogen regulated EGF receptor expression in those cells. These results suggest that estrogen may act through increasing production of TGF- α and regulation of the EGF receptor. Estrogen produced a concentration-related potentiation in the growth response to IGF-1 and EGF under conditions where the growth responses to EGF and IGF-1 were submaximal (Wimalasena *et al.*, 1993). Estrogen has been shown to exert its enhancement of EGF- and IGF-1-mediated growth through increased binding affinity for EGF receptor and IGF-1 receptor number (Wimalasena *et al.*, 1993). In other studies, estrogen caused a marked decrease in insulin-like growth factor binding protein-3 (IGFBP-3) mRNA, but increased IGFBP-5 mRNA levels, suggesting that IGFBP expression can be regulated in estrogen-responsive ovarian cancer cells by E2 (Krywicki *et al.*, 1993).

Germline mutations in the BRCA1 gene are associated with increased cancer risk in breast, ovary and prostate, but not in other tissues. The obvious implication, that BRCA1 mutations therefore affect neoplastic transformation in conjunction with hormonal factors, is supported by recent reports which showed that estrogen and prolactin stimulate proliferation of ovarian and breast carcinoma cells and concurrently upregulate BRCA1 mRNA and protein (Fan *et al.*, 1999). This demonstrates that in breast and prostate cancer cells, BRCA1 inhibits signaling by ligand-activated estrogen receptor ER-α and blocks its transcriptional activation function. Together, these data suggest that BRCA1 functions as a negative feedback inhibitor of growth induced by estrogen and prolactin. It is important to note that some ovarian carcinoma cells proliferate in response to estrogen (Kang *et al.*, 2001; Nash *et al.*, 1989) while normal OSE cells do not (Kang *et al.*, 2001; Karlan *et al.*, 1995).

2.3.4. Activin/Inhibin

Activin and inhibin are members of the TGF-β superfamily (Mathews, 1994; Vale *et al.*, 1988; Woodruff, 1998). Activin is a dimeric protein composed of two β subunits, βA-βA (activin A), βB-βB (activin B), or βA-βB (activin AB) (Vale *et al.*, 1988). Inhibin is composed of an α and one of two β subunits, α-βA (inhibin A) or α-βB (inhibin B). The main function of these gonadal peptides is to regulate FSH secretion from the anterior pituitary gland (Ling *et al.*, 1986; Vale *et al.*, 1986). However, since activin and inhibin are produced in the ovary (Eramaa *et al.*, 1993), it has been hypothesized that they may act via an autocrine/paracrine mechanism to regulate ovarian function (Eramaa *et al.*, 1993; Tuuri *et al.*, 1996). Activin mediates its cellular effects through heterodimeric complexes of type I and II activin serine/threonine kinase receptors (Eramaa *et al.*, 1995), which are expressed in normal and neoplastic OSE cells.

It has been demonstrated that recombinant activin has no mitogenic effect on normal OSE that also expresses activin receptors (Welt *et al.*, 1997). Interestingly, activin may function to support cell survival and stimulate the proliferation of epithelial ovarian carcinoma cell lines, including OVCAR-3, CaOV-3, CaOV-4, and SW-626 (Di Simone *et al.*, 1996, Fukuda *et al.*, 1998), whereas follistatin, an activin binding protein, inhibits this action (Fukuda *et al.*, 1998; Welt *et al.*, 1997). Most primary epithelial ovarian tumors synthesize and secrete activin *in vitro* and serum levels of activin are frequently elevated in women with epithelial ovarian cancer (Welt *et al.*, 1997). These findings suggested that, : 1) βA subunit mRNA is expressed, 2) activin is secreted more frequently than inhibin, 3) βA subunit mRNA expression is greater in neoplastic and normal epithelium after culture. Thus, activin may act as an autocrine/paracrine regulator of epithelial ovarian tumors, but its exact role in tumorigenesis has yet to be defined. Inhibin α subunit which was expressed in 47% cases of normal OSE, was not found in the epithelial component of ovarian cystadenomas, tumors of low malignant potential (LMP), or carcinomas. βA subunit was expressed in 93% cases of OSE, in the epithelial component of all cystadenomas, in 81% cases of LMP tumors and in 72% cases of carcinomas. These observations suggest that an imbalanced expression of inhibin and activin subunits in OSE may represent an early event that leads to epithelial proliferation (Zheng *et al.*, 1998).

Serum inhibin levels are elevated in most postmenopausal women with mucinous cystadenocarcinomas and mucinous borderline cystic types of epithelial ovarian tumors (Healy *et al.*, 1993), whereas immunoreactive inhibin is undetectable or present at low levels in normal postmenopausal subjects. The α-inhibin has been proposed to be a serum marker for epithelial ovarian cancer in postmenopausal women (Lambert-Messerlian *et al.*, 1997). Ovarian neoplasms may produce a variety of peptides related to the inhibin. It has been shown that inhibin B is

detected in more ovarian cancers than inhibin A (Robertson *et al.*, 1999). The majority of granulosa cell tumors appear to secrete significant amounts of dimeric inhibin-A, whereas mucinous tumors secrete predominantly other forms of inhibin, presumably related to the α-subunit (Burger *et al.*, 1996; 1998). Serous tumors may also secrete inhibin-related peptides but not dimeric inhibin-A (Burger *et al.*, 1996). The expression of inhibin subunit genes in granulosa cell tumors and in mucinous or serous epithelial ovarian tumors revealed that these tumors are the source of the increased immunoreactive inhibin observed in the serum of patients with ovarian tumors (Fuller *et al.*, 1999). On the contrary, it has also been reported that ovarian carcinomatous epithelial cells do not secrete inhibin and that serum inhibin levels detected in patients with epithelial ovarian carcinoma may reflect an ovarian stromal response to the ovarian carcinoma (Ala-Fossi *et al.*, 1999). Thus, the role of inhibin in ovarian cancer remains to be elucidated.

2.3.5. Growth factors

Trends in the expression and response to growth regulators include the secretion of, and responses to, factors found in the normal OSE (Berchuck *et al.*, 1993) as well as factors that may be typical of ovarian malignancies (Mills *et al.*, 1992). The former includes growth inhibition by TGF-β (Berchuck *et al.*, 1992) and growth stimulation by basic fibroblast growth factor (bFGF) (Di Blasio *et al.*, 1993), EGF and TGF-α (Kohler *et al.*, 1992).

2.3.5.1. Transforming growth factor-β

TGF-β is a multifunctional peptide that is involved in cell growth regulation, tissue remodeling, immune suppression and other crucial cellular functions *via* both autocrine and

paracrine mechanisms (Roberts and Sporn, 1990). Three mammalian TGF-β isoforms (TGF-β1, TGF-β2, and TGF-β3) that are encoded by different genes have been identified (Massague *et al.*, 1992). The peptides share extensive homology in amino acid sequence (70-80%), and exist as homodimeric chains of between 111 and 113 amino acids, with molecular weights of 25 kDa. Three types of receptors for TGF-β (TβRI, TβRII, TβRIII) that belong to the family of serine/threonine kinase membrane receptors have been identified (Massague, 1992; Wrana *et al.*, 1994). TGF-β binds to a type II TGF-β receptor (TβRII), which recruits and phosphorylates a type I TGF-β receptor (TβRI) (Bassing, 1994; Wrana *et al.*, 1992; 1994). TβRIII, also known as betaglycan, has no known signaling motif (Wrana *et al.*, 1992; 1994), and appears to bind and present TGF-β to TβRII (Carcamo *et al.*, 1995; Lopez-Casilla *et al.*, 1991; Wang *et al.*, 1991). The expression of TGF-β has been demonstrated in ovarian tumors, suggesting an autocrine and/or paracrine role of TGF-β (Bartlett *et al.*, 1992; 1997; Marth *et al.*, 1990). TGF-β inhibited the proliferation of monolayers of normal human ovarian epithelial cells by 40-70% (Berchuck *et al.*, 1992) and by 95% in primary epithelial ovarian cancer cell cultures obtained directly from ascites (Hurteau *et al.*, 1994). TGF-β inhibited colony formation of 7 of 9 fresh ovarian cancers in soft agar (Daniels *et al.*, 1989). In contrast, epithelial ovarian cancer cell lines are found to be relatively resistant to the growth inhibition of exogenous TGF-β treatment (Berchuck *et al.*, 1990; 1992). These data suggest that TGF-β may act as a growth inhibitor that prevents inappropriate proliferation of normal OSE cells, while loss of this autocrine inhibitory pathway may lead to cancer development *in vivo* and/or immortalization of cell *in vitro*.

Several possible mechanisms have been proposed to explain the loss of responsiveness to TGF-β in primary culture of ovarian carcinomas and/or ovarian cancer lines. Some cells may

become resistant to the effects of endogenous TGF-β because they cannot produce and/or activate secreted latent TGF-β. In this regard, it has been shown that normal ovarian epithelial cells can produce and activate TGF-β1 and 2, whereas production or activation does not occur in several ovarian cancer cell lines (Berchuck *et al.*, 1992). Like in other cells, defective ligand-binding to the cell surface caused by absence of TβRII or expression of a truncated form or splice variant of TβRII may account for the resistance to activated TGF-β in ovarian cancer cells (Inagaki *et al.*, 1993; Kadin *et al.*, 1994; Kalkhoven *et al.*, 1995 Park *et al.*, 1994; Wrana, 1992). It is also possible that alterations in signal transduction pathways may account for the development of resistance to TGF-β during the transformation process. In this regard, the binding of TGF-β to its cell surface receptors has been shown to down-regulate c-myc, a DNA-binding protein whose expression is induced by growth factors that stimulate proliferation (Pietenpol *et al.*, 1990). The loss of TGF-β responsiveness has been associated with the inability of TGF-β to down-regulate c-myc in some, but not all, cases of ovarian tumors (Fynan and Reiss, 1993). It has been suggested that inactivation of the p53 or Rb tumor suppressing gene products due to deletion, mutation, or binding of viral oncoproteins may be responsible for the loss of TGF-β responsiveness (Reiss *et al.*, 1993). However, in most ovarian cancers, it is thought that mutation and overexpression of p53 frequently occur, but this may not lead to the development of resistance to TGF-β (Hurteau *et al.*, 1994; Jacobs *et al.*, 1992; Kohler *et al.*, 1993).

The molecular mechanisms that mediate the growth inhibitory effect of TGF-β are poorly understood (Massague *et al.*, 1992). Binding of TGF-β to its receptors initiates a cascade of molecular events that are thought to decrease activity of cyclin-dependent kinase (CIP1/WAF1/p21), resulting in arrest of cell cycle from G1 into S phase of DNA synthesis in

normal and neoplastic ovarian cells (Massague *et al.*, 1992). In addition to the cell cycle inhibition, it has been shown that TGF-β can induce apoptosis in both normal and malignant cells under certain circumstances (Havrilesky *et al.*, 1995; Selvakumuran *et al.*, 1994). It is reported that malignant ovarian cells are more susceptible to apoptosis in response to TGF-β than their normal nontransformed counterparts (Havrilesky *et al.*, 1995).

2.3.5.2. Epidermal growth factor and transforming growth factor-α

The EGF receptor (also known as c-erbB1/HER1) is a membrane tyrosine kinase which forms homodimers after binding to either EGF or TGF-α (Ullrich and Schlessinger, 1990). Homodimerization activates tyrosine kinase activity and autophosphorylates several tyrosine moieties in the cytoplasmic domain of the receptor, thereby transmitting the growth stimulatory signal to the nucleus (Ullrich and Schlessinger, 1990). EGF receptors were detected in 33% to 75% of ovarian tumors using ligand binding, immunohistochemistry, or Northern blot analysis (Battagila *et al.*, 1989; Bauknecht *et al.*, 1988; 1993; Berchuck *et al.*, 1991; Henzen-Logmans, 1992; Kohler *et al.*, 1992; Morishige *et al.*, 1991; Owens *et al.*, 1991). The level of EGF receptor has been demonstrated to be higher in malignant ovarian tumors than in benign tumors or the normal ovary (Berns *et al.*, 1992; Owens *et al.*, 1993), implicating its prognostic importance. The contribution of a TGF-α/EGF receptor autocrine loop to the growth of epithelial ovarian cancer cells is corroborated by several studies. TGF-α levels in the normal ovary increase after menopause, i.e. at the peak incidence of ovarian neoplasms (Owens *et al.*, 1991; Owens and Leake, 1992). Exogenous treatment with TGF-α promotes the growth of several ovarian cancer cell lines *in vitro* and enhances direct clonogenic growth of ovarian tumor cells (Crew *et al.*, 1992; Kurachi *et al.*, 1991; Zhou and Leung, 1992). Co-expression of EGF receptor with TGF-

α, but not EGF, in primary ovarian tumors was reported (Morishige *et al.*, 1991). Neutralizing antibodies against either TGF-α or the EGF receptor induced growth inhibition in primary ovarian cancer cell cultures (Jindal *et al.*, 1994; Morishige *et al.*, 1991).

The amplification and/or over-expression of the c-erbB-2 (HER2/neu) oncogene product ($p185^{c\text{-}erbB\text{-}2}$), frequently observed in different types of tumors, was seen in 30-70% of human ovarian cancers (Haldane *et al.*, 1990; Slamon *et al.*, 1989), but in only 5-10% of normal ovarian cells (Hung *et al.*, 1992). At the mRNA level, c-erbB-2 has extensive homology with EGF receptor, c-erbB-3 and c-erbB-4 (Plowman *et al.*, 1993). Immunohistochemically, increased expression of c-erbB-3 and c-erbB-4 proteins has been demonstrated in malignant ovarian tumors as compared to benign ones (Simpson *et al.*, 1995). In spite of marked sequence homology between the EGF receptor and HER2, EGF and TGF-α do not bind to HER2 (Ullrich and Schlessinger, 1990). It has been demonstrated that HER2 can be transactivated by EGF through heterodimerization with EGF receptors (Goldman *et al.*, 1990) or by heregulin through heterodimerization with HER-3 or HER-4 receptors (Peles *et al.*, 1993). In addition to cell proliferation, EGFR and $p185^{c\text{-}erbB\text{-}2}$ activation has been shown to play an important role in cell motility (Wiechen *et al.*, 1999), which is mediated *in vitro* by several polypeptide growth factors, including HGF and EGF (Christen *et al.*, 1990; MacCawley *et al.*, 1998). In this regard, over-production of proteinases of the plasminogen activator (PA) and matrix metalloproteinase (MMP) families has previously been reported in ovarian cancer cells and tissues (Stack *et al.*, 1998). *In vitro*, EGF-dependent stimulation of migration, and induction of matrix metalloproteinase (MMP)-9 (gelatinase B) were observed in two ovarian cancer cell lines (OVEA6 and OVCA429) (Young *et al.*, 1996). These findings suggest that the EGF- or the

$p185^{c\text{-}erbB\text{-}2}$ -dependent enhancement of cell motility may contribute to peritoneal spread and invasion of tumor cells, resulting in tumor metastasis.

Treatment of an ovarian cancer cell line with a human-mouse chimeric anti-EGF receptor monoclonal antibody (mAb) or an anti-HER2 mAb resulted in growth inhibition (Ye Dingwei *et al.*, 1998). Concurrent treatment with two mAbs resulted in augmentation of inhibition. TGF-α-stimulated growth of ovarian cancer cell lines was completely inhibited by treatment with an EGF receptor-specific tyrosine kinase inhibitor, ZM252868, suggesting that blocking of receptor activation may have therapeutic value (Simpson *et al.*, 1999). The use of antisense molecules that are designed to specifically block encoded genetic information from sense DNA have been developed for targeting the c-erbB-2 oncogene. It has been shown that the c-erbB-2 antisense oligonucleotide to reduce $p185^{c\text{-}erbB\text{-}2}$ levels results in a growth inhibition of an ovarian cancer cell line (Wiechen K, Dietel M 1995 Wu *et al.*, 1996). Single-chain immunoglobulin (scFv) molecules that retain antigen-binding specificity but lack other functional domains have been designed to modulate the expression levels of oncogenes, the intracellular mobilization and function of oncoproteins. Introduction of anti-erbB-2-scFV resulted in down-regulation of cell surface erbB-2 gene expression and marked inhibition of cellular proliferation (Deshane *et al.*, 1994).

2.3.5.3. Hepatocyte growth factor

The HGF/Met system is considered to be a principal paracrine mediator of normal mesenchymal-epithelial interaction (Rosen *et al.*, 1994), and is also involved in the growth and spread of tumors (Jeffers, 1996). The Met/HGF receptor was overexpressed in a significant proportion of well-differentiated ovarian carcinomas (Di Renzo *et al.*, 1994; Huntsman *et al.*,

1999; Moghul *et al.*, 1994). Although little is known about the regulation of HGF and Met expression in ovarian tumors, the level of Met may be regulated by gonadotropin, steroids, certain cytokines and growth factors *in vivo* and in various cell lines (Hess *et al.*, 1999; Moghul *et al.*, 1994, Parrott and Skinner, 1998). HGF itself has been shown to autoregulate c-met mRNA levels (Boccaccio *et al.*, 1994; Moghul *et al.*, 1994). High levels of HGF are found in cystic fluids or ascites of ovarian cancer patients compared to the peritoneal fluid of normal women (Sowter *et al.*, 1999). Recombinant HGF increased migration and proliferation of ovarian cancer cell lines that express high levels of Met protein (Corps *et al.*, 1997; Ueoka *et al.*, 2000). Thus, high levels of Met expression in ovarian cancer cells may facilitate HGF-mediated tumor growth and dissemination (Corps *et al.*, 1997).

2.3.5.4. Insulin-like growth factors

Insulin-like growth factor (IGF) affects the growth and differentiation in normal and neoplastic cells (Cullen *et al.*, 1991; Daughaday, 1990; LeRoith *et al.*, 1995). IGF receptor-I (IGF-RI) mRNA was detected in ovarian cancer cell lines and primary or metastatic ovarian cancer tissues, suggesting a role of the IGF system in neoplastic ovarian cells (Beck *et al.*, 1994; van Dam *et al.*, 1994; Yee *et al.*, 1991). Expression of IGF-I, its receptor, and IGF-binding proteins (IGFBPs) in epithelial ovarian cancer cells and its mitogenic effect on these cells *in vitro* implicate a role for IGF-I in the regulation of human ovarian cancer (Conover *et al.*, 1998; Karasik *et al.*, 1994; Yee *et al.*, 1991). IGF-II is also expressed in both normal ovary and ovarian cancer and expression level of IGF-II is elevated in ovarian cancer (Yun *et al.*, 1996). The treatment of OVCAR-3 cells with hCG suppressed cisplatin-induced apoptosis via up-regulation of IGF-1 expression, suggesting that LH/hCG may influence the chemosensitivity of ovarian

cancer cells through an apoptotic-inhibitory signal (Kuroda *et al.*, 1998). In addition, the transformed ovarian mesothelial cells, overexpressed with IGF-RI, are resistant to apoptosis as a result of down-regulation of Fas expression (Coppola *et al.*, 1999). These results support the notion that the IGF system plays a role in tumor growth and apoptosis in ovarian cancer.

IGFBPs appear to bind to IGF and deliver them to target organs. There are a limited number of studies implicating the involvement of IGFBPs in ovarian cancer. IGFBP-2, the major binding protein in benign and malignant ovarian cancers, is highly expressed in malignant as compared to benign neoplasms (Flyvbjerg *et al.*, 1997; Kanety *et al.*, 1996), suggesting that IGFBP-2 may serve as a marker for ovarian cancer. Further, IGFBP-2 correlated positively with the serum tumor marker, CA 125. In contrast, the serum IGFBP-3 level was decreased in patients with ovarian cancer as shown by RIA and Western ligand blotting (Flyvbjerg *et al.*, 1997). Treatment with estradiol induced a marked decrease in IGFBP-3, but enhanced IGFBP-5 levels, indicating that IGFBP expression is differentially regulated by estradiol in estrogen-responsive ovarian cancer (Krywicki *et al.*, 1993).

Considering that IGFs, mediated by IGF-Rs, induce cell growth and mitogenesis in ovarian cancer, antisense or antibody therapy against IGFs and/or IGF-Rs can be considered as a potential management strategy of ovarian cancer patients. Treatment of cells with anti-sense IGF-I receptor oligonucleotides markedly inhibited cell proliferation (Muller *et al.*, 1998; Resnicoff *et al.*, 1993). Further, antisense oligonucleotide to IGF-II induced apoptosis in human ovarian cancer cells, suggesting that IGF-II may also be a potential target in the therapeutic treatment of ovarian cancer (Yin *et al.*, 1998).

2.3.5.5. Vascular endothelial growth factor

Angiogenesis is a critical phenomenon in the growth, progression, and metastasis of solid tumors. Vascular permeability factor/ vascular endothelial growth factor (VPF/VEGF) is a 34- to 50-kDa dimeric, disulfide-linked glycoprotein synthesized by normal and neoplastic cells (Berse *et al.*, 1992; Connolly *et al.*, 1989). Through binding to the specific membrane tyrosine kinase receptors that are expressed in vascular endothelial cells (Neufeld *et al.*, 1994), VEGF has been shown to be an important regulator of tumor angiogenesis. Abundant levels of VPF have been identified in the malignant effusions of ovarian tumors (Abu-Jawdeh *et al.*, 1996; Olson *et al.*, 1994; Yeo *et al.*, 1993), indicating that VPF may be an important mediator of ascites formation and tumor metastasis observed in the neoplastic ovary. The expression of VEGF mRNA and protein (Abu-Jawdeh *et al.*, 1996; Boocock *et al.*, 1995; Olson *et al.*, 1994) has been demonstrated in ovarian carcinoma, suggesting that neoplastic OSE is one source of VEGF production. *In vitro*, the conditioned medium from VEGF-positive ovarian cancer cell lines has been shown to stimulate DNA synthesis of vascular endothelium (Olson *et al.*, 1994). *In vivo*, treatment of mice carrying tumor engraftment with a function-blocking VEGF antibody (A4.6.1) specific for human VEGF significantly inhibited subcutaneous SKOV-3 tumor growth as compared with controls (Mesiano *et al.*, 1998). In mice bearing intraperitoneal tumors, ascites production and intraperitoneal carcinomatosis were completely inhibited by the treatment with a VEGF antibody (Mesiano *et al.*, 1998). These results suggest that neutralization of VEGF activity may have clinical application in inhibiting malignant ascites formation in ovarian cancer. Angiogenesis has been correlated with prognosis in patients with ovarian cancer. Higher positive immunostaining for VEGF and serum VEGF levels were observed in women with ovarian carcinoma than in tumors of low malignant potential (LMP) and benign cystadenoma (Yamaoto

et al., 1997). High VEGF expression in epithelial ovarian carcinomas was found to be associated with poor overall survival (Hartenbach *et al.*, 1997). Serum VEGF levels decreased after surgical removal of tumor in ovarian cancer patients, suggesting that serum VEGF could be used as a marker for monitoring tumor progression and ascites formation (Gadducci *et al.*, 1999; Oehler and Caffier, 1999; Tempfer *et al.*, 1998; Yamaoto *et al.*, 1997).

2.3.5.6. Other growth factors

Platelet-derived growth factor (PDGF) is a dimeric protein composed of two related A- and B- chain polypeptides encoded by separate genes. Two distinct receptors for PDGF have been found according to affinity (PDGF-Rα and PDGF-Rβ). A functional role of PDGF via autocrine growth stimulation has been suggested. Expression of PDGF and PDGF-Rα in ovarian tumor cells is related to progression of malignant ovarian tumors, suggesting an independent role for PDGF-Rα as a prognostic factor (Henriksen *et al.*, 1993). However, a contradictory report showed that many ovarian carcinomas lose the PDGF receptors, while PDGF stimulates growth of normal OSE cells, which have both α and β receptors (Dabrow *et al.*, 1998). The loss of PDGF-Rα and PDGF-Rβ may be indicative of independence from hormonal influences to cell growth. Platelet-derived endothelial cell growth factor (PD-ECGF) is associated with angiogenesis and the progression of human ovarian cancer. The levels of PD-ECGF and its mRNA were higher in ovarian cancers than in normal ovaries, suggesting that PD-ECGF might be related to advanced stages of ovarian cancers associated with neovascularization (Fujimoto *et al.*, 1998). Thus, prevention of angiogenic activity by PD-ECGF may have a potential role in ovarian tumor therapeutics (Reynolds *et al.*, 1994).

Basic FGF (bFGF) and other members of the FGF family share several biological properties that have the potential to mediate neoplastic cell growth. It has been shown that ovarian cancer cell lines produce and respond to bFGF and other members of the FGF family (Crickard *et al.*, 1994). The bFGF and its receptor are also expressed in epithelial ovarian tumors (Di Blasio *et al.*, 1995). In advanced primary ovarian tumors, the levels of bFGF mRNA and protein were significantly higher regardless of histological types (Fujimoto *et al.*, 1997), indicating that this growth factor may contribute to growth, invasion and metastasis with neovascularization. It is hypothesized that bFGF may induce a fibroblastic response, which causes tumors with a high bFGF to be less aggressive than those with less stromal tissues (Obermair *et al.*, 1998).

2.3.6. Cytokines

While the secretion of cytokines is a normal OSE function (Ziltener *et al.*, 1993), their recruitment into autocrine loops may be important during neoplastic progression. Cytokines produced by and growth stimulatory for ovarian carcinomas include M-CSF (Kacinski *et al.*, 1990), GM-CSF (Cimoli *et al.*, 1991), IL-1 and IL-6 (Malik and Balkwill, 1991; Scambia *et al.*, 1994) and TNF-α (Balkwill, 1992; Bast *et al.*, 1993; Marth *et al.*, 1996; Wu *et al.*, 1992; 1993). High levels of M-CSF and IL-6 in blood and ascitic fluid correlate with a poor prognosis in ovarian cancer, as does overexpression of the M-CSF receptor *fms* (Kacinski *et al.*, 1990). Interestingly, *fms* is expressed by many ovarian cancers but not by benign ovarian tumors (Kacinski *et al.*, 1990) or normal OSE (Berchuck *et al.*, 1993). Thus, M-CSF, when secreted by normal OSE, acts in a paracrine manner but becomes an autocrine regulatory factor with malignant progression. GM-CSF is a regulatory glycoprotein that stimulates the production of granulocytes and macrophages. Recombinant human GM-CSF stimulates colony formation in

human ovarian cancer cell lines, IGROV-1, A2774, ME-180, Pa-1 and A2780 (Cimoli *et al.*, 1991).

IL-1 and IL-6 enhance tumor cell motility and metastasis (Malik and Balkwill, 1991) and cause changes in gene expression including the induction of TNF-α which is mitogenic for OSE cells but growth inhibitory for ovarian cancer cells (Marth *et al.*, 1996). Stimulation of proliferation by IL-1β could be partially blocked by an antibody against TNF-α or by soluble TNF-α receptor (Wu *et al.*, 1993). Thus, TNF-α may function as an autocrine/paracrine growth factor in normal and malignant ovarian epithelial cells. Epithelial ovarian cancer cells produce IL-6, a multifunctional cytokine with diverse biological effects, in both ovarian cancer cell lines and primary ovarian tumor cultures (Watson *et al.*, 1990). IL-6 may be a useful tumor marker in some patients with epithelial ovarian cancer, as it correlates with tumor burden, clinical disease status, and survival (Berek *et al.*, 1991). Inhibition of IL-6 gene expression by exposure to IL-6 antisense oligonucleotides resulted in greatly decreased cellular proliferation (Watson *et al.*, 1993). However, the addition of exogenous IL-6 failed to restore the proliferation of the antisense-treated cells and antibodies to IL-6 did not consistently inhibit cell growth (Watson *et al.*, 1990), suggesting that IL-6 is not an autocrine growth factor for these established ovarian tumor cell lines. As the majority of epithelial ovarian cancers produce IL-6, the direct specific inhibition of IL-6 gene expression may be of potential therapeutic value (Watson *et al.*, 1993). Many of these agents are produced normally by various ovarian cell types and by cells of the immune system that reside in the ovary. Factors from these sources may contribute to the metaplastic and neoplastic changes in the OSE.

Interferon-γ (IFN-γ) is known to modulate many cellular functions. A clinical relevance of IFN-γ has been suggested because IFN-γ has an antiproliferative activity on the majority of the

established human ovarian carcinoma cell lines (Mobus *et al.*, 1993). It has been shown that IFN-γ decreases constitutive tyrosine phosphorylation of erbB-2 and inhibits erbB-2 kinase activity in an ovarian cancer cell line, SKOV3 cells, which over-expresses erbB-2 (Mishra and Hamburger, 1994). The elevated expression of tumor-associated antigens and major histocompatibility complex (MHC) antigens by IFN-γ may improve immunogenicity of ovarian tumor cells and explain the therapeutic effects observed in IFN therapy of ovarian cancer (Mobus *et al.*, 1993).

2.3.7. Lysophosphatidic acid

A potent growth stimulatory factor from ascites of ovarian cancer patients has been purified and characterized as ovarian cancer activating factor (OCAF), which plays a role in ovarian tumorigenesis both *in vitro* and *in vivo* (Xu *et al.*, 1995, Fang *et al.*, 2000). In addition, this purified OCAF induced proliferation of ovarian cancer cells. OCAF is composed of various species of lysophosphatidic acid (LPA), including LPAs with polyunsaturated fatty acyl chains (linoleic, arachidonic, and docosahexaenoic acids) (Xu *et al.*, 1995). LPA is a bioactive phospholipid with mitogenic and growth factor-like activities that acts via specific cell-surface receptors present in many normal and transformed cell types. LPA has been implicated as a growth factor present in ascites of ovarian cancer patients (Westermann *et al.*, 1998).

As reviewed above, multiple factors including peptide hormones, sex steroids, growth factors and cytokines have been implicated as stimulatory or inhibitory growth regulators in ovarian cancer. These regulators appear to exert their actions through specific receptors in an endocrine, paracrine or autocrine manner. A better understanding of the potential cross-talk between these

regulatory pathways in normal and neoplastic OSE cells will be a necessary first step in the understanding of ovarian tumorigenesis.

2.5. Apoptosis and bcl-2 gene family

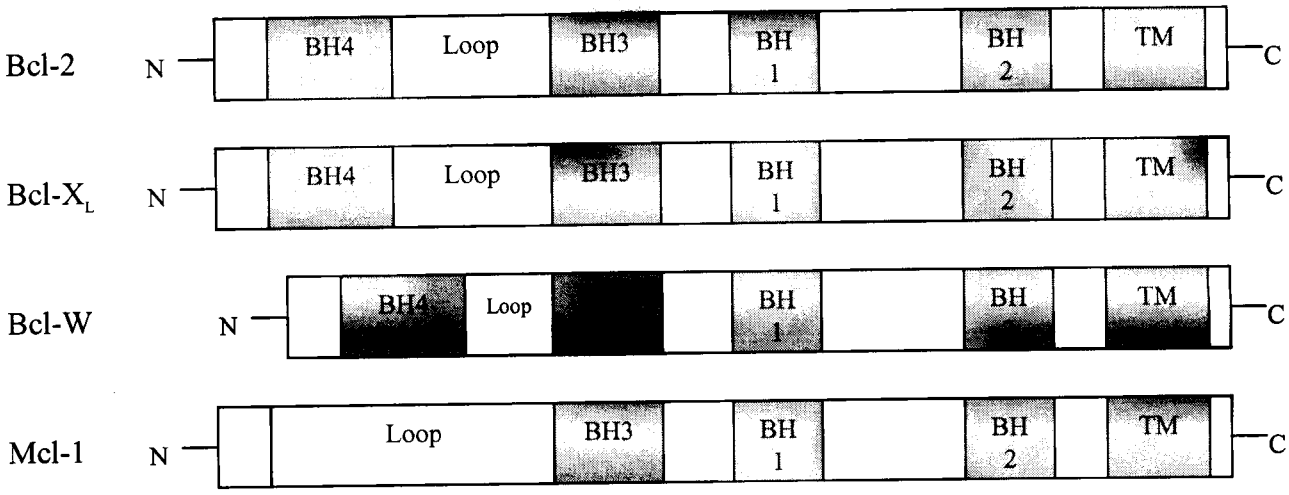
2.5.1. Apoptosis and cancer

Until recently, cancer was considered as a disease of abnormal cell proliferation. However, developments within the cell death field over the past ten years have provided a new prospective on how cell proliferation is normally maintained at equilibrium and unveiled how abnormalities in cell death regulation can contribute to the progression and development of malignancy. Apoptosis, which is documented as programmed cell death (PCD), plays a pivotal role in the normal development and homeostasis of all multicellular organisms. Deregulation of this process, resulting in either too much or too little cell death, may cause developmental defects and a wide range of disease status. The purpose of this process is to remove unwanted host cells. Apoptosis occurs in three situations: 1) for development and homeostasis, 2) as a defense mechanism, and 3) in aging (Vaux and Strasser, 1996). Morphologically, this phenomenon is characterized by chromatin condensation, membrane blebbing and loss of cell volume (Chao DT, Korsmeyer SJ 1998; Minn *et al.*, 1998). In general, single cell surrounded by viable neighbors may be susceptible to apoptosis. Apoptosis is a genetically programmed mode of cell death that is regulated by genes, including oncogenes and oncosuppressor genes, which may be mutated, deleted or abnormally expressed in neoplasms, thus altering tumor cell susceptibility to apoptosis (Martin, 1997).

2.4.2. Bcl-2 family

The 131 somatic cells undergoing programmed cell death among 1090 somatic cells are generated during the development of the nematode *Caenorhabditis elegans*. Genetic analysis revealed that three genes, CED-3, and CED-4 and CED-9 control all 131 of these developmental programmed cell death (Hengartner and Horvitz, 1994). A mammalian homolog of CED-9 was identified as bcl-2 at the inter-chromosomal breakpoint of the t(14;18), the molecular hallmark of follicular B cell lymphoma (Bakhshi *et al.*, 1985; Tsujimoto *et al.*, 1985). The discovery that bcl-2 plays a role in preventing apoptosis instead of promoting proliferation established a new concept of oncogenes (Hockenbery *et al.*, 1990; Korsmeyer, 1992; Vaux *et al.*, 1988). The identification of multiple bcl-2 homologs which form homodimers or heterodimers suggests that these molecules function at least in part through protein-protein interactions (Chao and Korsmeyer, 1998). The first pro-apoptotic homolog, bax, is a 21-kDa protein that shares homology with bcl-2 in conserved regions including BH-1 (bcl-2 homology region-1) and BH-2 (Fig. 4). It has been demonstrated that bax heterodimerizes with bcl-2 and homodimerizes with bax itself (Oltvai *et al.*, 1993). When bax was overexpressed in cells, apoptotic death in response to a death signal was accelerated, however, when bcl-2 was overexpressed, it heterodimerized with bax and the death signal was repressed (Oltvai *et al.*, 1993). Thus, the ratio of bcl-2 to bax is critical in determining susceptibility to apoptosis. Bax is widely expressed in tissues, including a number of sites in which cells die during normal maturation (Krajewski *et al.*, 1994; Oltvai *et al.*, 1993). The family has further expanded to include the death antagonists bcl-2, bcl-X_L, bcl-W, Mcl-1 as well as the pro-apoptotic molecules bax, bcl-X_S, BAK and BAD (Fig. 4). The bcl-X gene gives rise to two mRNA species by alternative splicing, producing two protein products: bcl-X_L,

Anti-apoptotic



Pro-apoptotic

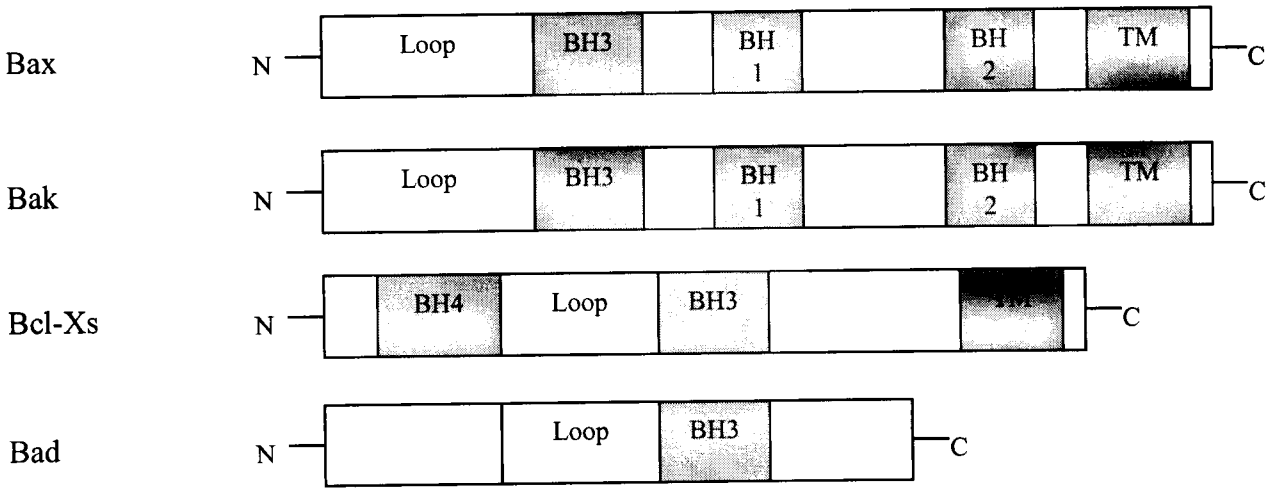


Figure 4. Summary of anti-apoptotic and pro-apoptotic bcl-2 family members. Bcl-2 homology regions (BH1 -BH4) are indicated with shared boxes. TM: transmembrane domain

endowed with death repressor activity, and a shorter variant, bcl-X_S, which functions as a dominant inhibitor of bcl-2, thus inducing apoptosis (Boise *et al.*, 1993). Mutational analysis of bcl-2 and bcl-X_L identified key residues within BH1 and BH2 domains required for both heterodimerization with bax and repression of cell death (Yin *et al.*, 1994). The overall ratio of the death agonists to antagonists determines the susceptibility to a death signal. Conserved domains BH1, BH2 and BH3 participate in the formation of various dimer pairs as well as the regulation of cell death (Sedlak *et al.*, 1995). Another mitochondrial component implicated in apoptotic cell death is the release of cytochrome-c from the inter membrane space. Release of cytochrome c into the cytosol is a strong activator of caspases (Kluck *et al.*, 1997; Yang *et al.*, 1997). When bcl-2 prevented apoptosis, cytochrome c was not released. It has been suggested that cytochrome c release may precede a fall in mitochondrial membrane potential.

The CED-3 was cloned and demonstrated to be homologous to a previously cloned mammalian gene, interleukin-1β (IL-1β) converting enzyme (ICE) (Miura *et al.*, 1993). The ICE was initially identified as a cysteine protease that converts the 31-kDa pro form of IL-1β into a 17.5-kDa mature form. It was confirmed that ICE plays a critical role in programmed cell death when ICE was transiently introduced into a mammalian cell line and found to induce apoptosis. Many more ICE-like proteases have been cloned in mammalian cells, and these proteases have been renamed caspases (Faucheu *et al.*, 1995; Kamens *et al.*, 1995; Kumar *et al.*, 1994). Transfection of many of these caspases into mammalian cells induces apoptosis, and elimination of these caspases in mice by gene targeting causes defects in apoptosis (Kuida *et al.*, 1995; 1996). All of these caspases are synthesized in a pro form and activated by proteolytic cleavage (Kumar, 1995). The poly(ADP)-ribose polymerase (Lazebnik *et al.*, 1994), nuclear lamins (Takahashi *et al.*, 1996), fodrin (Cryns *et al.*, 1996), p21-activated kinases 2 (PAK2) (Rudel and

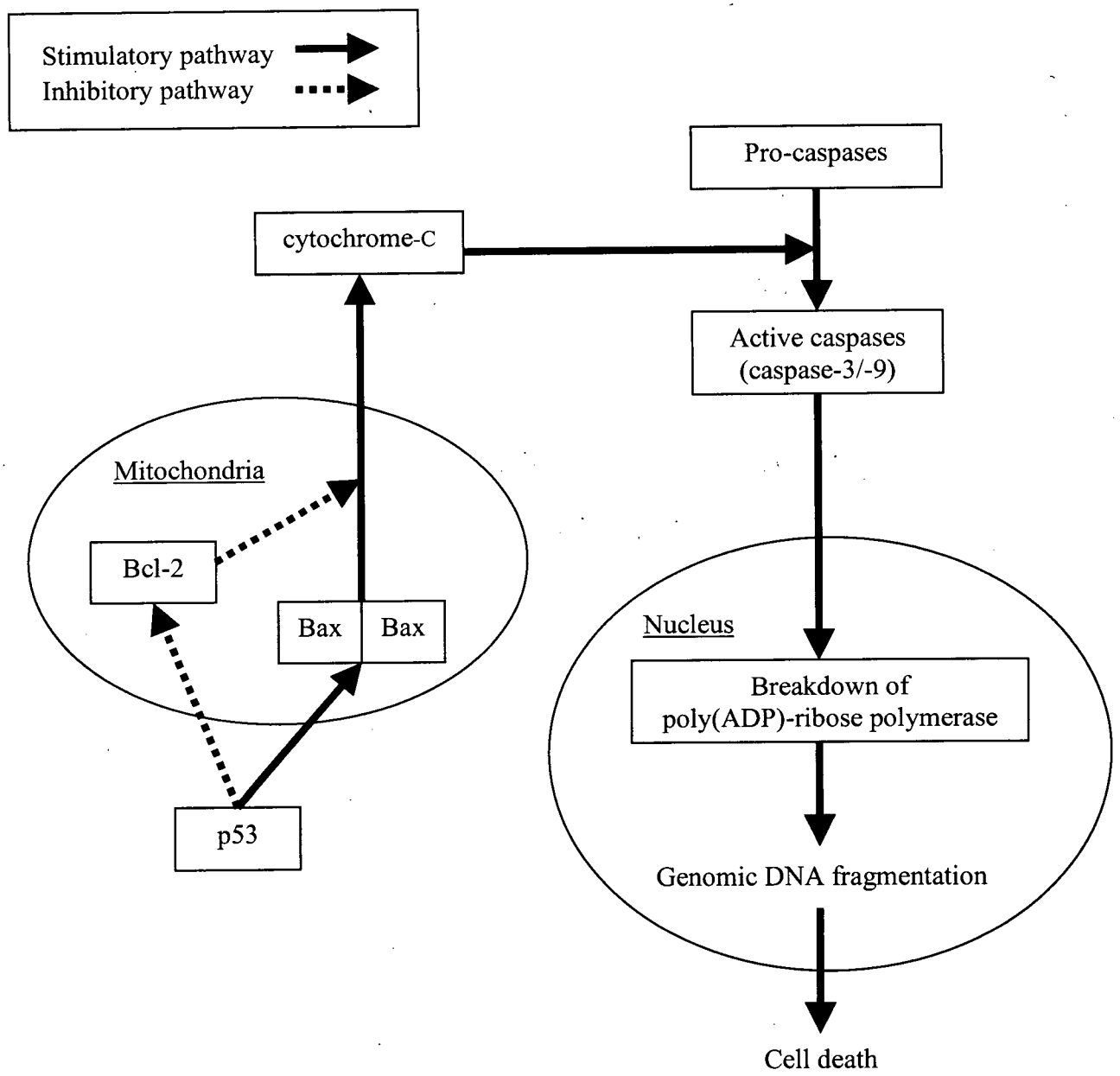


Figure 5. Activation of apoptotic pathway through homodimerization (bax/bax) of pro-apoptotic bcl-2 gene family, release of cytochrome-c, and activation of caspases.

Bokoch, 1997), and DNA fragmentation factor (DFF) (Liu *et al.*, 1997) have been suggested as the targets for caspases. Thus, it has been proposed that cleavage of these substrates induces programmed cell death (Fig. 5) and is responsible for at least some of the morphological changes associated with apoptosis. Further, the studies have been extended to investigate the critical role of caspases in programmed cell death on multiple mammalian cell death receptors such as TNF receptor family, including TNF receptor-1 and Fas/APO-1 (Yuan, 1997). The cytoplasmic domain of these receptors contains an amino acid region known as a death domain. In addition, these death receptors play a role in apoptosis through a direct link to the downstream pathway of caspases, which is responsible for inducing apoptosis (Boldin *et al.*, 1996).

2.4.3. Regulation of apoptosis and bcl-2 gene family

The bcl-2 family constitutes a critical intracellular checkpoint of apoptosis within a common cell death pathway and these apoptotic proteins are widely accepted as regulators of cell death (Chao and Korsmeyer, 1998; Minn *et al.*, 1998). Understanding the regulation of apoptosis in cancer cells has recently become an area of intense research. Overexpression of bcl-2 has been demonstrated to provide protection against a wide variety of death-inducing signals such as growth factor deprivation, loss of cell attachment to ECM proteins, oncogenes, tumor suppressor genes, cytotoxic T cells, radiation and essentially all available chemotherapeutic drugs (Reed, 1994). In contrast, overexpression of bax was reported to accelerate apoptosis by inhibiting the death repressor activity of bcl-2, probably by forming bcl-2-bax complexes or by competing with other bcl-2 targets (Oltvai *et al.*; 1993). In addition, physiological and pathological stimuli, such as steroids (Bu *et al.*, 1997; Perillo *et al.*, 2000; Wang and Phang, 1995) or peptide hormones (Imai *et al.*, 1998; Kuroda *et al.*, 1998), growth factors (Havrilesky *et al.*, 1995; Lafon *et al.*,

1996), cytokines (Gooch, 1998), radiation (Filippovich *et al.*, 1997) and anticancer drugs (Judson *et al.*, 1999 Strobel *et al.*, 1996; Zaffaroni *et al.*, 1998) have been demonstrated to regulate apoptotic pathways in ovarian or breast cancer cells, suggesting that these factors may play a role in regulating pro- or anti-apoptotic genes in these cells.

Bcl-2 inhibits programmed cell death triggered by several stimuli. It has been demonstrated that bcl-2 may inhibit apoptosis induced by glucocorticoids (Alnemri *et al.*, 1992) and by anticancer drugs, such as 5-fluorodeoxyuridine or taxol (Fisher *et al.*, 1993) in leukemic cells. The activity of bcl-2 has been shown to block gamma-radiation induced cell death (Sentman *et al.*, 1991). The mechanism of apoptosis prevention has not been elucidated, but it has been suggested that bcl-2 may function in an antioxidant pathway which inhibits lipid peroxidation (Hockenbery *et al.*, 1993) and/or mitochondrial electron and metabolite transport (Hockenbery *et al.*, 1990). Its main effect is to prolong cell survival by avoidance of apoptosis.

2.5. Mitogen-Activated Protein Kinases

The mitogen-activated protein kinases (MAPKs) are a group of serine/threonine kinases that are activated in response to a diverse array of extracellular stimuli, and mediate signal transduction from the cell surface to the nucleus (Davis, 1994). Extracellular signal-regulated kinases (ERK1 and ERK2), *c-jun* terminal kinase/stress-activated protein kinases (JNK/SAPK), and p38 are three of the best characterized MAPK family members (Cobb and Goldsmith, 1995; Fanger, 1999) (Fig. 6). Other MAPK family members, ERK3, 4 and 5, four p38-like kinases, and p57 MAPK have been cloned, but a biological role for them has not been elucidated yet (Fanger, 1999). It is well known that the MAPK cascade is activated *via* two distinct classes of cell surface receptors, receptor tyrosine kinases (RTKs) and G protein-coupled receptors (GPCRs).

The signals transmitted through this cascade lead to activation of a set of molecules that regulate cell growth, division and differentiation. The most extensively studied members of the cascade are the ERK1 (p44 MAPK) and ERK2 (p42 MAPK). ERK1 and/or ERK2 are activated by mitogenic stimuli, whereas JNK/SAPK and p38 are activated in response to stress such as heat shock, osmotic shock, cytokines, protein synthesis inhibitors, antioxidants, ultra-violet, and DNA-damaging agents (Garrington and Johnson, 1999; Robinson and Cobb, 1997). MAPK family members are directly regulated by kinases known as MAPK kinases (MAPKKs), which activate the MAPKs by phosphorylation of tyrosine and threonine residues (Fanger, 1999; Robinson and Cobb, 1997). Currently, seven different MAPKKs have been cloned and characterized (Fanger, 1997). The first MAPKKs cloned were MAPK/ERK kinase 1 and 2 (MEK 1/2), which specifically activate ERKs. MKK3 and 6 specifically activate p38, whereas MKK5 stimulates the phosphorylation of ERK5. The MKK4 and 7 are known to activate JNK. The MAPKKs are activated by a rapidly expanding group of kinases called MAPKK kinases (MAPKKKs), which activate the MAPKKs by phosphorylation of serine and threonine residues (Fanger, 1999; Robinson and Cobb, 1997). These include Raf-1, A-Raf, B-raf, MAPK/ERK kinase 1-4 (MEKK1-4), apoptosis-stimulating kinase-1 (ASK-1), and mixed lineage kinse-3 (MLK-3). The MAPKKKs may be activated by kinases known as MAPKKK kinases (MAPKKKKs), one of which is p21-activated kinase (PAK). In addition, low molecular weight GTP-binding (LMWG) proteins regulate the activity of MAPKKKs and MAPKKKKs (Fanger, 1999). There are several different families of LMWG proteins, two of which include the Ras (N-Ras, K-Ras, and H-Ras) and Rho (Rac 1, 2 and 3, Cdc42 and Rho A, B and C) families. Activated MAPKs have been shown to phosphorylate a large number of both cytoplasmic and nuclear proteins, exerting their specific functions. For example, activated ERK1/2 phosphorylate

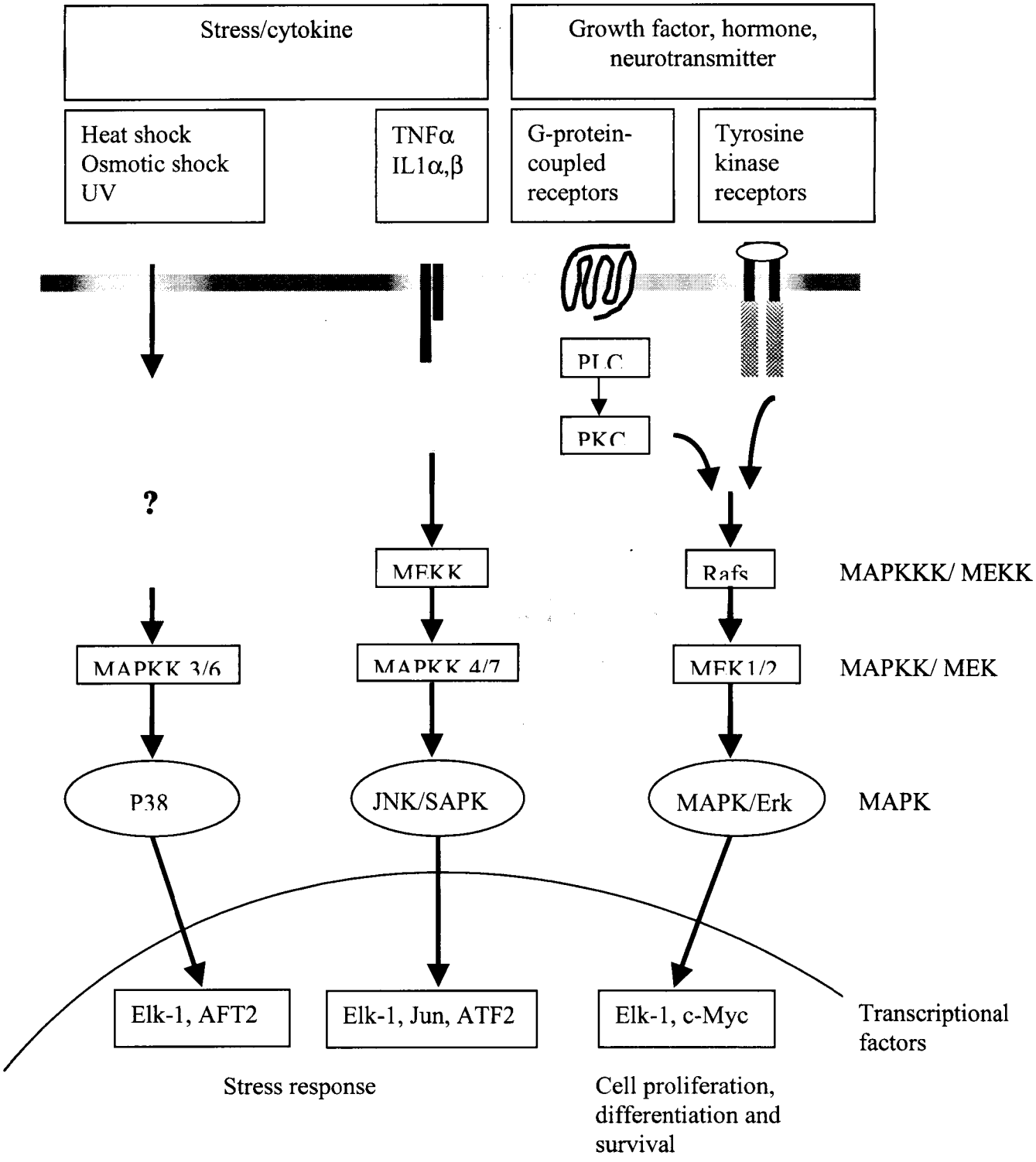


Figure 6. Signal-transduction pathways of receptors or stress-activated MAPKs.

ternary complex factor (TCF) proteins such as Elk-1 and SAP-1, which form transcriptional complexes with serum response factor (SRF) in the promoter region of early response genes (e.g. c-fos, egr-1, junB) and thereby regulate their expression (Wasylyk *et al.*, 1998) (Fig. 6). Many of these nuclear proteins, as a result of their ability to modulate expression of other proteins, are potential candidates for critical factors involved in the cellular response to stimuli.

As mentioned earlier, the MAPK cascade can be activated *via* both RTKs and GPCRs, which include FSH and GnRH receptors. In ovarian cancer cells, MAPKs were regulated by cisplatin (Persons *et al.*, 1999), paclitaxel (Wang *et al.*, 1999), endothelin-1 (Vecca *et al.*, 2000) and GnRH (Kimura *et al.*, 1999) suggesting that the MAPK signaling pathway plays an important role in the regulation of proliferation, survival and apoptosis in response to these external stimuli.

3. Rationale and Objectives

The common epithelial ovarian tumors appear to arise from the ovarian surface epithelium (OSE), which is a simple squamous-to-cuboidal mesothelium covering the ovary, as mentioned earlier (Auersperg *et al.*, 1998; Herbst, 1994; Nicosia *et al.*, 1991). The exact mechanism of ovarian tumorigenesis is not known even though this disease accounts for the most common cancer among gynecological malignancies. The incessant ovulation theory was suggested, whereby repeated ovulation contributes to (pre)neoplastic change of OSE, suggesting that the wound healing process of ruptured OSE may play a role in the disease in women (Fathalla, 1971). Therefore, endocrine and autocrine factors including hormones and multiple growth factors were suggested to influence the occurrence of ovarian tumors during the menstrual cycle (Godwin *et al.*, 1993; Hamilton, 1992; Piver *et al.*, 1991; Rao and Slotman, 1991 Risch, 1998; Shoham, 1994; Westerman *et al.*, 1997). Therefore, we tested the hypothesis that endocrine and autocrine

factors may play a role in the induction of proliferation and/or apoptosis related with bcl-2 and bax expression in normal and neoplastic OSE cells during the development of ovarian cancer.

Specific aim 1: To investigate the differential expression of activin/inhibin subunit and activin receptor mRNAs (*EXPERIMENT A*)

The importance of the activin/inhibin system in regulating cell proliferation and possibly ovarian tumor development has been suggested recently (Di Simone *et al.*, 1996; Fukuda *et al.*, 1998; Welt *et al.*, 1997). A study of six human epithelial ovarian cancer cell lines revealed the expression of βA and/or βB subunits as well as activin type II receptor. Several ovarian cancer cell lines produced activin *in vitro* and exogenous activin induced proliferation of these cells (Di Simone *et al.*, 1996). However, the exact role of activin and its receptors has not been elucidated. Therefore, this study was designed to further examine the autocrine role of activin in normal and neoplastic OSE cells, *via* determining the expression of activin/inhibin subunits and activin type II receptors, and its effect on cellular proliferation.

Specific aim 2: To determine the effect of activin and TGF-β on the regulation of apoptosis (*EXPERIMENT B*)

The TGF-β superfamily plays a critical role in the regulation of proliferation and apoptosis for ovarian tumorigenesis. The importance of activin, a member of the TGF--β superfamily, in regulating cell proliferation and possibly ovarian tumor development has been suggested (Di Simone *et al.*, 1996; Fukuda *et al.*, 1998; Welt *et al.*, 1997). Therefore, activin appears to act as an autocrine/paracrine factor in epithelial ovarian tumors, but its role in tumorigenesis has yet to be defined (Welt *et al.*, 1997). In addition, it has been demonstrated that TGF-β inhibits

proliferation but does not induce apoptosis in normal OSE cells. In contrast, TGF-β induces apoptosis in some ovarian tumors that are growth-inhibited by TGF-β (Havrilesky *et al.*, 1995).

This study was performed to investigate the role of activin and TGF-β in normal, early neoplastic and tumorigenic OSE cells. The expression of the activin/inhibin subunits and activin receptors was determined. In addition, the effects on cell number and induction of apoptosis by activin and TGF-β were also examined. Finally, the regulation of pro-apoptotic bax and anti-apoptotic bcl-2 was investigated following treatment with activin and TGF-β.

Specific aim 3: To examine the role of E2 on the regulation of anti-apoptotic bcl-2 mRNA and protein (*EXPERIMENT C*)

The actions of estrogen are mediated through interactions with its intracellular receptor, a member of the steroid/thyroid/retinoid receptor gene superfamily (Tsai and O'Malley, 1994). The classical estrogen receptor (ER, now referred to as ERα) was thought to be the only form of nuclear receptor able to bind estrogen, and mediate its hormonal effects in their target tissues. However, the cloning of a second form of estrogen receptor, now referred to as ERβ, has prompted a reexamination of the estrogen signaling system (Mosselman *et al.*, 1996). Recent studies have revealed different tissue distributions and expression levels of ERα and ERβ in the ovary, suggesting different biological roles of ERα and ERβ in this tissue (Kuiper *et al.*, 1996; Mosselman *et al.*, 1996; Sar and Welsch, 1999). In addition, the existence of ERα and ERβ in normal OSE and ovarian cancers has been demonstrated (Brandenberger *et al.*, 1998; Lau *et al.*, 1999). While E2 is not a mitogen for normal OSE (Karlan *et al.*, 1995), treatments with exogenous estrogen resulted in growth stimulation of several ER-positive ovarian carcinoma cell

lines *in vitro* (Chien *et al.*, 1994; Galtier-Dercure *et al.*, 1992; Langon *et al.*, 1994). However, the role of estrogen in ovarian tumorigenesis and regulation of apoptosis by estrogen in neoplastic OSE cells remains uncertain.

The present study was performed to investigate the role of E2 in the regulation of apoptosis in normal, early neoplastic, tumorigenic and late neoplastic OSE cells. The expression of ERα and ERβ was determined in these OSE cell lines to investigate the effect of E2. Furthermore, cell proliferation and prevention of apoptosis by E2 were examined in immortalized OSE cell lines. Finally, to elucidate the mechanism of E2 in the prevention of apoptosis, the regulation of pro-apoptotic bax and anti-apoptotic bcl-2 was investigated following treatments with E2 and/or anti-estrogen, tamoxifen.

Specific aim 4: To investigate the effect of FSH on activation of mitogen-activated protein kinase (*EXPERIMENT D*)

In addition to its well-established function in reproductive physiology, follicle-stimulating hormone (FSH) has been implicated in ovarian cancer development (Konishi *et al.*, 1999; Zheng *et al.*, 2000). Epidemiological studies demonstrated an increased occurrence of ovarian cancer with exposure to high levels of gonadotropins during postmenopause or infertility therapy (Risch, 1998 Shushan *et al.*, 1996; Whittemore *et al.*, 1992). Expression of FSH receptor (FSH-R), a G protein-coupled receptor, has been demonstrated in normal OSE (Zheng *et al.*, 1996), ovarian inclusions and epithelial tumors (Zheng *et al.*, 2000), implicating a possible role of FSH in these cells. In addition, treatment of FSH resulted in growth-stimulation of ovarian cancer cells (Wimalasena *et al.*, 1992; Zheng *et al.*, 2000) in a dose- and time-dependent manner *in vitro*. Despite these findings, the precise molecular mechanism of FSH in terms of growth stimulation

and intracellular signaling in ovarian cancer remains unknown. A number of factors including cisplatin (Persons *et al.*, 1999), paclitaxel (Wang *et al.*, 1999), endothelin-1 (Vecca *et al.*, 2000) and GnRH (Kimura *et al.*, 1999) have been shown to regulate MAPK activity on ovarian cancer cells, suggesting a possible signaling pathway in the regulation of growth and differentiation. The effect of FSH on growth stimulation in ovarian cancer cells may be mediated *via* the MAPK signaling cascade.

Little is known about the molecular events that mediate FSH actions in normal and neoplastic ovarian cells. Considering that FSH plays a role in these cells, the effect of FSH in normal OSE and IOSE cell lines was investigated. In the present study, experiments were designed to examine 1) the expression of FSH-R at the mRNA level in normal OSE and IOSE cell lines, 2) the proliferative effect of FSH in these cells, and 3) the effect of FSH on ERK1/2 activation.

Specific aim 5: To elucidate the expression and apoptotic role of the second form of gonadotropin-releasing hormone (GnRH-II) (*EXPERIMENT E*)

In addition to its well-documented role in the regulation of gonadotropin synthesis and secretion in the reproductive hormone cascade, GnRH, a hypothalamic decapeptide, has been suggested to act as an autocrine/paracrine regulator in normal ovarian surface epithelium (Kang *et al.*, 2000). GnRH and its synthetic analogs have a direct growth-inhibitory effect on ovarian tumors (Emons *et al.*, 1993a; Yano *et al.*, 1994). This concept is based on the detection of binding sites for GnRH, as well as the expression of GnRH and its receptor gene transcripts in these tumors. Recently, a second form of GnRH with characteristics of chicken GnRH-II, now referred to as GnRH-II (pGln-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly) has been cloned in brain extracts from rhesus monkeys (Lescheid *et al.*, 1997). In addition, GnRH-II has been

demonstrated to encode a different gene and is expressed at significantly higher levels outside the brain, including kidney (30-fold higher than in any brain region), bone marrow, and prostate, suggesting that GnRH-II may have multiple functions in various tissues (White *et al.*, 1998). Interestingly, GnRH-II has been shown to bind the GnRH receptor (GnRH-R) up to 100 times more effectively than GnRH-I, suggesting GnRH-II may act *via* GnRH-R outside the brain (King and Millar, 1991). However, the role of GnRH-II in normal and neoplastic OSE cells remains to be elucidated.

To examine the potential role of GnRH-II as a possible regulator, it has been investigated for the first time the expression of GnRH-II in normal and neoplastic OSE cells. In addition, to investigate the physiological significance, the direct receptor-mediated growth regulatory effect of GnRH-I and –II on neoplastic OSE cells was investigated for the first time in this study. Finally, to examine the regulatory effects of GnRH-I and –II on apoptosis, the induction of cell death was investigated following treatment with exogenous GnRH-I and –II.

II. MATERIALS AND METHODS

1. Materials

Human recombinant activin A (rh-activin A), follistatin and follicle-stimulating hormone (FSH) were generously provided by Dr. A. F. Parlow in National Hormone & Pituitary Program of Harbor-UCLA Medical Center (Torrance, CA). Recombinant TGF-β1, 17β-estradiol, tamoxifen, staurosporin, 3-isobutyl-1-methylxanthine (IBMX), and (D-Ala^6)-GnRH (GnRH agonist) were purchased from Sigma-Aldrich Corp. (Oakville, Canada). PD98059, a MAPK/ERK kinase (MEK) inhibitor, was purchased from New England Biolabs Inc. (Berverly, MA). GnRH-II was purchased from Peninsula Laboratories (Belmont, CA).

2. Cell cultures

2.1. Normal OSE cells

Normal OSE cells were scraped from the ovarian surface during laparoscopies for nonmalignant disorders and cultured as previously described (Kruk *et al.*, 1990) in medium 199:MCDB 105 (Sigma-Aldrich Corp., Oakville, Canada, respectively) containing 10% FBS, 100 U/ml penicillin G and 100 μg/ml streptomycin (Life Technologies, Inc., Burlington, Canada) in a humidified atmosphere of 5% CO_2-95% air and passaged with 0.06% trypsin (1:250)/0.01% EDTA in Mg^{2+} /Ca^{2+} - free HBSS when confluent. The human ovarian epithelial carcinoma cell line, OVCAR-3 was cultured in medium 199:MCDB 105 containing 10% FBS, 100 U/ml penicillin G and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2-95% air.

2.2 Primary cultured ovarian tumors (PC-OVC) from surgical specimens in women

Primary ovarian tumors and ascite fluid from ovarian cancer patients were obtained from Vancouver General Hospital and British Columbia Cancer Agency, respectively. Solid tissue was processed within 1 hr after surgery in medium199:MCDB 105 supplemented with 100 U/ml penicillin G and 100 μg/ml streptomycin as previously described (Crickard *et al.*, 1983). Areas of fat, nontumor, and obviously necrotic tissue were removed from the tumor mass and minced with a scalpel into 2 mm^3 pieces. The minced tissue was incubated in medium199:MCDB 105 containing 4mg/ml collagenase(type III) and 0.1 mg/ml bovine pancreatic DNase (Sigma Co.) for 1 hr in 37 C CO_2 incubator. The suspension was mixed with a pipet, and the cells were passed through a fine mesh in the presence of medium199:MCDB 105 containing 20% FBS, 100 U/ml penicillin G, 100 μg/ml streptomycin, and 2.5 μg/ml Fungizone. The cell suspension was layered onto Ficoll paque (Amersham-Pharmacia Co.) and centrifuged to remove red blood cells. The cancer cells were placed on a 10 cm plastic culture dish with 10 ml of medium199:MCDB 105 supplemented with 20% FBS for 1 to 2 h. Fibroblasts and mesothelial cells tend to attach to the plastic substrate during this period, leaving a relatively pure preparation of tumor cells in suspension. The tumor cells could be preplated on plastic substrate with subsequent passages to eliminate any fibroblasts present. The suspended tumor cells were centrifuged at 500 x g for 10 min and resuspended in complete medium. The tumor cells were maintained on culture dishes in the presence of medium199:MCDB 105 supplemented with 20% FBS, 100 U/ml penicillin G, and 100 ug/ml streptomycin. Ascites fluid was processed and maintained as previously described (Hurteau *et al.*, 1994). Briefly, cancer cells were removed from the fluid by centrifugation. The cells were layered onto Ficoll paque and centrifuged to remove red blood cells. The cancer cells were placed in medium199:MCDB 105 supplemented with 10% FBS for 24 h. The suspended

tumor cells were pelleted, resuspended and maintained on culture dishes in the presence of medium199:MCDB 105 supplemented with 20% FBS, 100 U/ml penicillin G, and 100 μg/ml streptomycin.

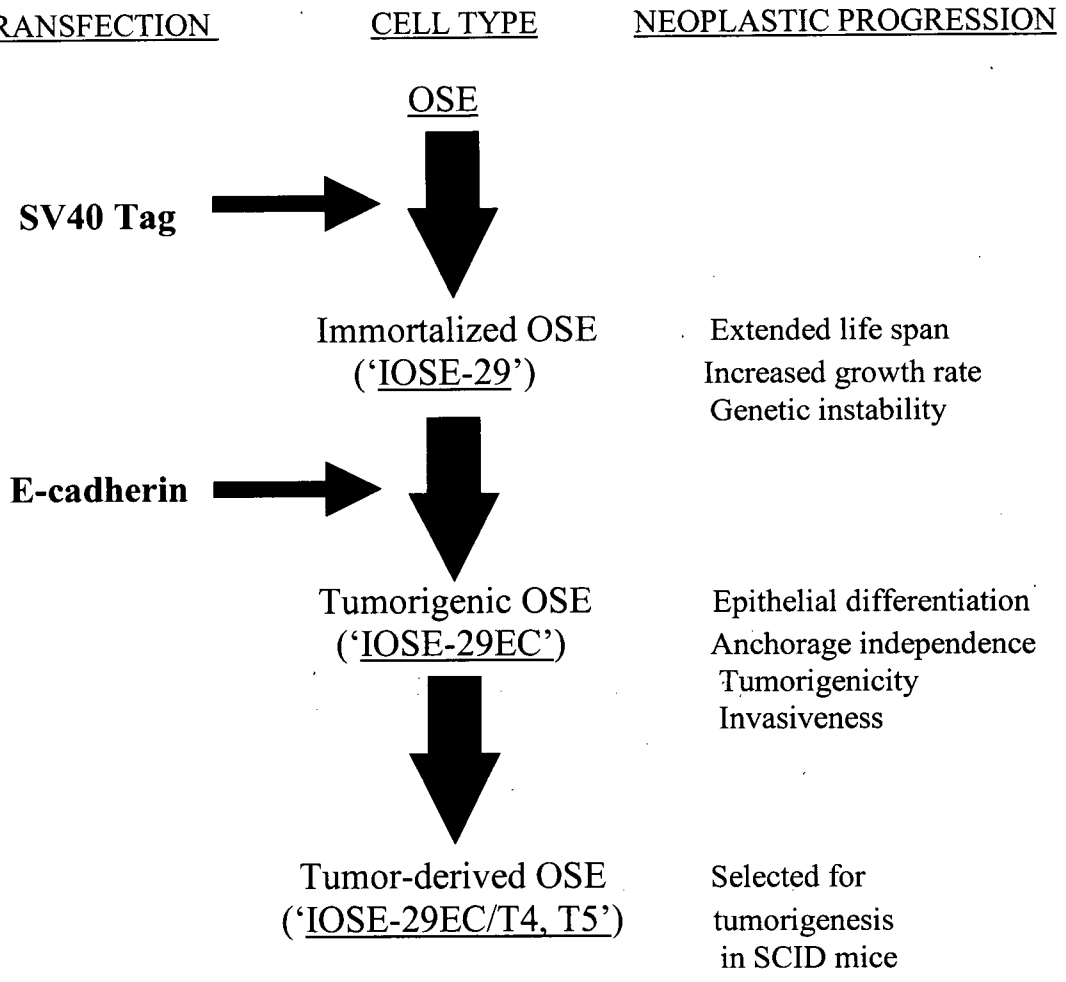
2.3. Immortalized ovarian surface epithelium cell lines (IOSE cell lines)

As outlined in Table 1, a culture system with cells representing several stages in the neoplastic progression of OSE has been developed. The IOSE-29 cell line (referred to as IOSE-Mar in some previous publications) was generated by transfection with the immortalizing simian virus 40 early genes into normal human OSE at passage 5 (Maines-Bandiera *et al.*, 1992). The IOSE-29EC cell line was made from IOSE-29 at passage 11 by transfecting the full length mouse E-cadherin cDNA under the control of the β-actin promoter (Auersperg *et al.*, 1999). The IOSE-29EC/T4 and IOSE-29EC/T5 were established from tumors that arose in IOSE-29EC-inoculated SCID mice, and they formed tumors on mesenteries and omentum, invaded the liver and thigh musculature, and produced ascites (Ong *et al.*, 2000). For monolayer culture, all cell lines were maintained on tissue culture dishes (Corning; Corning Laboratory Sciences Co.) in a 1:1 mixture of medium 199 / MCDB 105 medium supplemented with 10% FBS, 100 U/ml penicillin G and 100 μg/ml streptomycin.

2.4. Established epithelial ovarian cancer cell lines

OVCAR-3, the epithelial ovarian cancer cell line derived from adenocarcinoma was kindly provided by Dr. T.C. Hamilton, Fox Chase Cancer Center, Philadelphia, PA (Chien *et al.*, 1994). Epithelial ovarian cancer cell lines including CaOV-3, OVCAR-3 and SKOV-3 cells were cultured under the conditions as described above.

TABLE 1. Development of early neoplastic, tumorigenic and late neoplastic OSE cells from normal human OSE



3. Treatments

To study the basal expression level of activin/inhibin subunits and activin type II receptors, 2 $\times 10^5$ normal OSE and OVCAR-3 cells were plated and cultured on 35-mm culture dishes in the above-mentioned medium. In a dose-response experiment, 2×10^5 OVCAR-3 cells were cultured in 35 mm dish for 48 h and treated with rh-activin A (1, 10 and 100 ng/ml) for 24 h. To investigate the specificity of activin, the cells were treated with activin (10 ng/ml) plus follistatin (100 ng/ml), which is a specific activin binding protein. In a time-course experiment for activin, 2×10^5 OVCAR-3 cells were treated with 10 ng/ml rh-activin A for 3, 6, 12, and 24 h.

To study the regulation of pro-apoptotic bax and anti-apoptotic bcl-2 mRNA by 17β-estradiol (E2, Sigma-Aldrich Corp., Oakville, Canada), 2×10^5 IOSE-29EC cells were plated onto 35-mm culture dishes. After a preincubation of 48 h, the cells were treated with E2 at concentrations of 10^{-8}, 10^{-7} and 10^{-6} M in phenol-red free medium 199 (Sigma-Aldrich Corp.) with 2 % charcoal/dextran treated FBS (HyClone Laboratories Inc.) for 24 h. To confirm the specificity of E2, the cells were treated with E2 (10^{-7} M) plus tamoxifen (10^{-6} M, Sigma-Aldrich Corp.) for 24 h. Control cultures were treated with vehicle (absolute ethyl alcohol). Furthermore, to investigate the regulation of bax and bcl-2 apoptotic proteins by E2, 2×10^5 IOSE-29EC cells were plated onto 35-mm culture dishes and cultured for 48 h. Subsequently, the cells were treated with E2 (10^{-8}, 10^{-7} and 10^{-6} M) plus tamoxifen (10^{-6} M) for 48 h.

4. RNA extraction and RT-PCR procedures

Total RNA was prepared from cultured cells using the RNaid kit (Bio/Can Scientific, Mississauga, Canada) according to the manufacturer's suggested procedure. RNA integrity was confirmed by agarose gel electrophoresis and ethidium bromide staining. The total RNA

concentration was determined from spectrophotometric analysis at $A_{260/280}$. Complementary DNA (cDNA) was synthesized from 2.5 μg total RNA by reverse transcription at 37 C for 2 h using a first strand cDNA synthesis kit (Pharmacia Ltd., Uppsala, Sweden). The synthesized cDNA was used as template for polymerase chain reaction (PCR) amplification. The amplification was achieved using a thermal cycler (DNA Thermal Cycler, Perkin-Elmer, Norwalk, CT). Total RNA (2.5 μg) was reverse transcribed into first strand cDNA (Amersham Pharmacia Biotech, Oakville, Canada), following the manufacturer's procedure. Synthetic oligonucleotides used for PCR primers and PCR conditions are listed in Table 2 based on the published sequences. The PCR reactions were performed in 25 μl PCR mixture containing 1 X PCR buffer, 0.2 mM each dNTP, 1.6 mM $MgCl_2$, 50 pmol specific primers, each cDNA template, and 0.25 unit *Taq* polymerase. The PCR reaction was performed for two or three independent cDNA preparations of each RNA sample. Twelve microliters of PCR products were analyzed by agarose (1%) gel electrophoresis and visualized by ethidium bromide staining, and the sizes were estimated by comparison to DNA molecular weight markers.

5. Southern blot analysis

Following electrophoresis, PCR products were transferred to nylon membranes (Hybond-N, Amersham Co.) and fixed by UV irradiation. The blotted membranes were prehybridized for 3 h at 42 C in prehybridization solution containing 50 % formamide, 5X SSC, 0.1 % N-lauroyl sarcosine, 0.02 % SDS, and 2 % blocking solution. The prehybridized membranes were hybridized overnight at 42 C with digoxigenin-labeled probes. The cDNA clones for α, βA, and βB subunits were subcloned from human granulosa cells and verified by sequencing.

TABLE 2. Oligonucleotide sequences of PCR primers for human

Oligo		Sequence	Cycle No.
α subunit	sense	5'-CCC AGT TTC ATC TTC CAC TAC-3'	27
	antisense	5'-CCC ATA GGC ACA GAA GTG AA-3'	
βA subunit	sense	5'-AGG TCA ACA TCT GCT GTA AG-3'	27
	antisense	5'-TTC TCT GGA CAA CTC TTG CT-3'	
βB subunit	sense	5'-TGT TGC AGG CAA CAG TTC TTC-3'	27
	antisense	5'-GAA TGA CTG TAC TTA GCC CAC-3'	
ActR-IA	sense	5'-GAT GAG AAG TCA TGG TTC AGG-3'	30
	antisense	5'-TAT GTT TGG CCT TTG TTG ATC-3'	
ActR-IB	sense	5'-CTG GCT GTC CGT CAT GAT GCA-3'	40
	antisense	5'-CAA TTC GCT CTC AGA GTC TCC-3'	
ActR-IIA	sense	5'-ACC AGT GTT GAT GTG GAT CTT-3'	30
	antisense	5'-TAC AGG TCC ATC TGC AGC AGT-3'	
ActR-IIB	sense	5'-TTC TGC TGC TGT GAA GGC AAC-3'	30
	antisense	5'-GAG GTC GCT CCT CAG CAA TAC-3'	
ERα	sense	5'-ATG ACC ATG ACC CTC AAC ACC AA-3'	30
	antisense	5'-CTT GGC AGA TTC CAT AGC CAT AC-3'	
ERβ	sense	5'-TAC AGC ATT CCC AGC AAT GTC AC-3'	30
	antisense	5'-GAA GTG AGC ATC CCT CTT TGA AC-3'	
Bax-α	sense	5'-ATG GAC GGG TCC GGG GAG CAG C-3'	24
	antisense	5'-CCC CAG TTG AAG TTG CCG TCA G-3'	
Bcl-2	sense	5'-GGT GCC ACC TGT GGT CCA CCT G-3'	30
	antisense	5'-CTT CAC TTG TGG CCC AGA TAG G-3'	
FSH-R	sense	5'-GAG AGC AAG GTG ACA GAG ATTC C-3'	30
	antisense	5'-CCT TTT GGA GAG AAT GAA TCT T-3'	
GnRH-II	sense	5'-GCC CAC CTT GGA CCC TCAGAG-3'	30
	antisense	5'-CGG AGA ACC TCA CAC TTT ATT GG-3'	
GnRH-R	sense	5'-GTA TGC TGG AGA GTT ACT CTG CA-3'	33
	antisense,	5'-GGA TGA TGA AGA GGC AGC TGA AG-3'	
β-actin	sense	5'-GGA CCT GAC TGA CTA CCT CAT GAA-3'	15
	antisense	5'-TGA TCC ACA TCT GCT GGA AGG TGG-3'	
GAPDH	sense	5'-ATG TTC GTC ATG GGT GTG AAC CA-3'	18
	antisense	5'-TGG CAG GTT TTT CTA GAC GGC AG-3'	

The cDNA clones for activin receptor IA and IB were provided by Dr. C. Peng (York Univ., Toronto, Canada). The cDNA clones for activin receptor IIA and IIB were kindly provided by Dr. W.Vale (The Salk Institute, La Jolla, CA) and Dr. C. Peng, respectively. The cDNA clones for bax and bcl-2 were subcloned from ovarian cancer cell line (OVCAR-3) and verified by sequence analysis. PCR products of ERα and ERβ isolated from human granulosa cells were cloned into pCRII vector using the TA Cloning Kit (Invitrogen, San Diego, CA) and were sequenced by the dideoxy nucleotide chain termination method using the T7 DNA polymerase sequencing kit (Amersham Pharmacia Biotech.). The cDNA clones for bax and bcl-2 were subcloned from ovarian cancer cell line (OVCAR-3) and verified by sequence analysis above-mentioned. The cDNA clone for FSH-R was kindly provided by Dr. Minegishi (Gunma University School of Medicine, Gunma, Japan) (Minegish *et al.*, 1991). The cDNA probes were labeled with digoxigenin cDNA labeling kit (Roche Molecular Biochemicals, Laval, Canada) for hybridization. The hybridized membranes were detected with luminescence method (Boehringer Mannheim Co.) and exposed to X-ray film for 1 to 10 min at room temperature. The specific bands were scanned and quantified using a computerized visual light densitometer (model 620, BioRad Laboratories, Richmond, CA).

6. Cloning and sequencing

PCR products of α, βA, βB subunits, bax, bcl-2, GnRH-II were subsequently cloned using TA cloning kit (Invitrogen, San Diego, CA) and sequenced to verify their identities using T7 sequencing kit (Amersham Pharmacia Biotech.). All sequence analysis was performed using both M13 universal forward and reverse primers. Double-strand DNA templates (2 μg in 32 μl) were denatured for 10 min by adding 8 μl of 2M NaOH in reaction volume of 40 μl. Following

incubation, DNA was precipitated with a sodium acetate/ethanol (7 µl of 3 M sodium acetate, pH 4.8, 4 µl of distilled water, and 120 µl absolute ethanol) and collected by centrifugation (10, 000 x g, 20 min), washed with 70% ethanol, and dried under vacuum. The denatured DNA was resuspended in 10 µl of distilled water, combined with 2 µl of primer, and 2 µl of annealing buffer (1 M Tris-HCl, pH 7.6, 100 mM $MgCl_2$, 160 mM DTT). The reaction was incubated for 5 min at 65 C and then for 10 min at 37 C. After incubation, the reaction mixture was allowed to cool down to room temperature for 15 min. Following the annealing reaction, 3 µl of Labeling Mix, 5 µCi of [α-^{35}S]dATP (Amersham Pharmacia Biotech.), and 2 µl of diluted T7 DNA polymerase (3 unit) were added and incubated for 5 min at room temperature. Four of 2.5 µl dNTP mix (840 µl M of A, C, G, and T mix) were prepared and prewarmed for 5 min before addition of 4.5 µl of the labeling reaction to each mix. After incubation for 5 min at 37 C, reactions were stopped with 5 µl of Stop Solution (0.3 % each bromophenol blue and xylene cyanol FF, 10 mM EDTA (pH 7.5), and 97.5% deionized formamide) and boiled for 5 min prior to loading (2.5 µl) onto a sequencing gel. Polyacrylamide 6%/7M urea sequencing gels were prepared and prewarmed at 45 W constant power for 1 h. The samples were loaded and run for 3-4 h at 45 W constant power. Subsequently, gels were dried at 80 C for 2 h using a gel dryer (Model 583, Biorad Laboratories, Richmond, CA) and exposed to autoradiography at –70 C for 8-24 h. Each PCR product was verified to be identical to the published sequence (GenBank, NIH) through the website (www.ncbi.nlm.nih.gov).

7. Northern blot analysis

Total RNAs (50- 100 µg) were denatured in 50 % formamide/2.2 M formaldehyde, incubated at 60 C for 15 min, and electrophoresed on 1% denaturing agarose gel (20 mM MOPS, 2.2 M

formaldehyde, 8 mM sodium acetate, 1 mM EDTA pH 8.0) at a constant 70 voltage for 3-4 h. A capillary transfer of RNA to nylon membrane was performed overnight in 10X SSC, as described in Southern blot analysis. The membrane was then irradiated by UV light for 5 min to cross-link the RNA to membrane. Radioactive labeled cDNA probe of FSH-R was prepared from Ramdon Labeling Kit (Life Technologies Inc.) according to the manufacturer's suggested procedure. DNA templates (100 ng) were denatured at 100 C for 5 min and placed on ice. Two µl each of following dNTP (dCTP, dGTP, and dTTP), 15 µl of random priming buffer, 50 µCi of [α-^{32}P]dATP (3000 Ci/mmol, Amersham Pharmacia Biotech.) and 3 unit of Klenow fragment were mixed. After 3 h incubation period at 25 C, the reaction was stopped by adding 5 µl of Stop Buffer (0.2 mM Na_2EDTA, pH 7.5). The labeled DNA was purified using G-50 Sephadex column (Amersham Pharmacia Biotech.). The membrane was pre-hybridized in standard hybridization solution (50% formamide, 5X SSPE, 5X Denhardt's, 0.5 % SDS, 100 µg/ml denatured herring sperm DNA) for 3 h at 42 C. The membrane was washed once with 2X SSC/0.1 % SDS at room temperature and twice with 0.1 X SSC/0.1% SDS at 65 C for 15 min. After washing, the membrane was exposed to Kodak Omat x-ray film (Eastman Kodak Co.).

8. Immunoblot analysis

The immortalized ovarian surface epithelium cells (IOSE-29, IOSE-29EC, IOSE-29EC/T4, and IOSE-29EC/T5) were seeded at a density of 2×10^5 cells in 35 mm culture dishes and cultured in a humidified atmosphere of 5 % CO_2-95 % air at 37 C. Cells were washed twice with ice-cold PBS and lysed in ice-cold RIPA buffer (150 mM NaCl, 1 % Nonidet P-40, 0.5 % deoxycholate, 0.1 % SDS, 50 mM Tris (pH, 7.5), and 1 mM PMSF, 10 µg/ml leupeptin, 100 µg/ml aprotinin). The extracts were placed on ice for 15 min and centrifuged to remove cellular

debris. Protein content of the supernatants was determined using a Bradford assay (Bio-Rad Laboratories) (Fig. 7). Twenty-five or 30 μg of total protein was run on 10 % SDS-PAGE gels and electrotransferred to a nitrocellulose membrane (Amersham Pharmacia Biotech.). The membranes were immunoblotted using rabbit polyclonal antibodies of activin receptor IA, IB, IIA, and IIB provided by Dr. W. Vale (The Salk Institute, La Jolla, CA) or a mouse monoclonal antibody for ERα (Santa Cruz Biotech., Santa Crus, CA) and a goat polyclonal antibody for ERβ (Santa Cruz Biotech.). To determine if the *in vitro* treatments affected the expression of the genes involved in apoptosis, the membranes were immunoblotted using mouse monoclonal antibodies of bax (BD Pharmingen Inc., Mississauga, ON, Canada) and bcl-2 (SantaCruz Biotech.). The loaded amount of protein was normalized with actin protein in the same membrane. In addition, the membrane was immunoblotted using a mouse monoclonal antibody specific to the phosphorylated p44/p42 MAPK (P-MAPK, Thr^{202}/Tyr^{204}) (New England Biolabs Inc.). Alternatively, a membrane was probed with a rabbit polyclonal antibody for p44/42 MAPK (New England Biolabs Inc.), which detects total MAPK (T-MAPK, phosphorylation-state independent) levels. After washing, the signals were detected with horseradish peroxidase-conjugated secondary antibody, and visualized using the ECL chemiluminescent system (Amersham Pharmacia Biotech.).

9. [^{3}H]thymidine incorporation assay

Normal OSE and OVCAR-3 cells were plated in 24-well plates at 1×10^4 cells/well in 0.5 ml medium 199:MCDB 105 supplemented with 10 % FBS and antibiotics for 24 h. After a preincubation period, the cells were treated with rh-activin A (1, 10, and 100 ng/ml) and/or follistatin (100 ng/ml) in medium plus 2% FBS as previously described (Di Simone *et al.*, 1996; Wang *et al.*, 1996a). After 48 h, the medium was removed, and the cells were treated with the

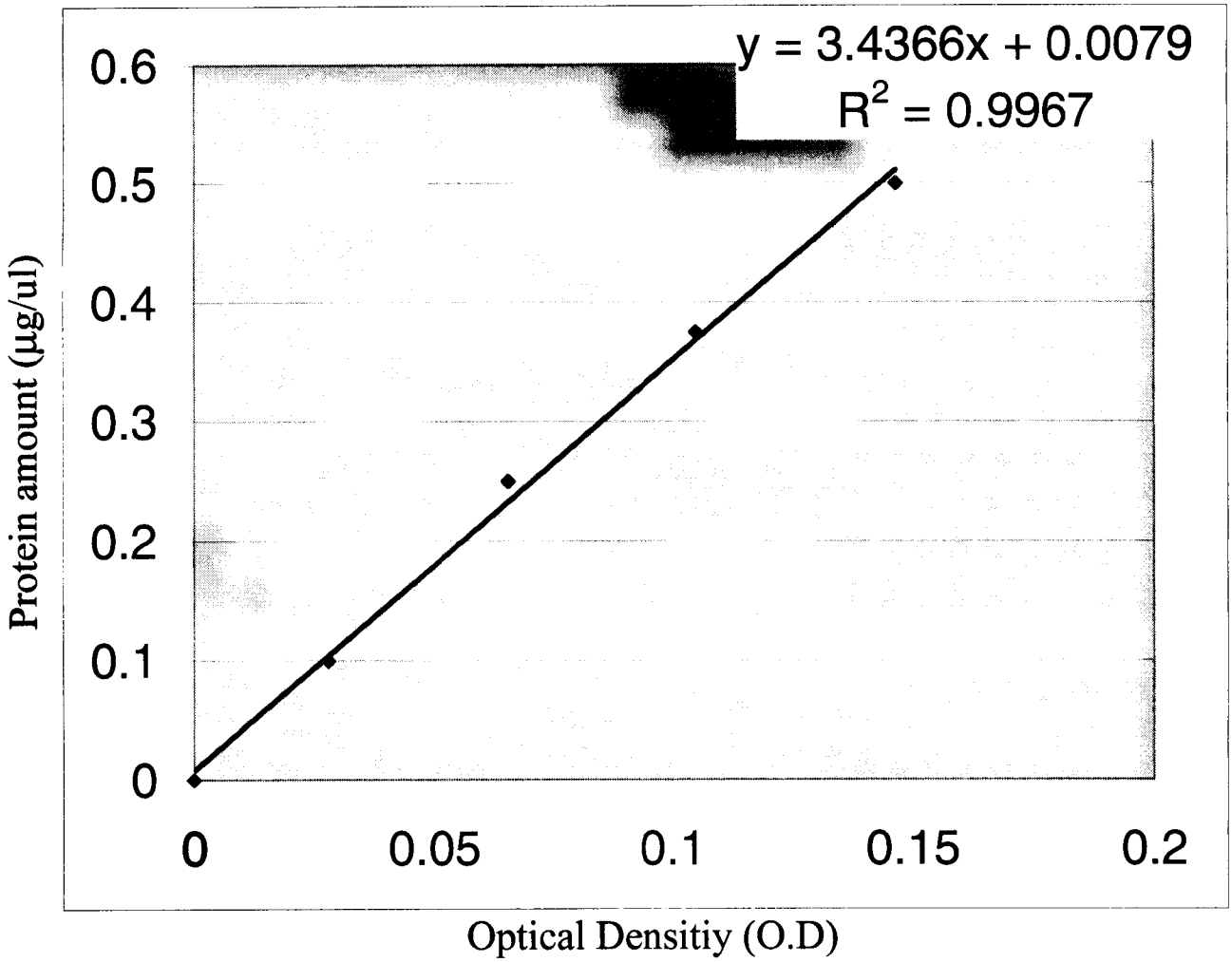


Figure 7. A standard curve for the protein assay. Cellular lysate (10 µl) was incubated with 25 µl of Reagent A and 200 µl of Reagent B, for 15 min with gentle shaking at room temperature. An absorbance of the samples was measured using ELISA reader at 630 nm wavelength.

same concentration of rh-activin A for an additional 48 h. After 96 h incubation, the medium was removed again, the cells were cultured in the presence of the same concentration of rh-activin A and 1 μCi[^{3}H]thymidine (5.0 Ci/mmol; Amersham Inc.) for another 24 h. Human recombinant follistatin was used to block the effect of activin. For TGF-β treatment, the cells were incubated with 0.1, 1, 10 ng/ml TGF-β and replaced daily for 72 h. During the last 6 h, 1 μCi[^{3}H]thymidine and the same concentration of TGF-β were added to each well as previously described (Havrilesky *et al.*, 1995).

On the day of treatment, the cells were incubated with increasing concentrations (10^{-8}, 10^{-7} or 10^{-6} M) of E2 in phenol-red free medium 199 with charcoal/dextran treated FBS for 2-6 days. On the days indicated in the results, during the last 6 h of the incubations to be harvested, the medium was changed to include the same concentration of E2 and 1 μCi[^{3}H]thymidine. Tamoxifen (10^{-6} M, Aldrich-Sigma Corp.), a specific E2 antagonist, was used to block the E2 effect. In addition, the cells were incubated with increasing concentrations (10, 100 and 1000 ng/ml) of FSH and 1 μCi[^{3}H]thymidine in serum free media for 24 h. In this study, the cells were treated with increasing concentrations (10^{-9}, 10^{-8} and 10^{-7} M) of GnRH agonist, (D-Ala^6)-GnRH or GnRH-II everyday for 6 days. To block the effect of the GnRH agonist, the cells were treated with the (D-Ala^6)-GnRH (10^{-7} M) or GnRH-II plus antide (10^{-7} M, Sigma-Aldrich Corp.) at equimolar concentration for 6 days.

After the culture medium was removed, to determine [^{3}H]thymidine incorporation, the cells were washed three times with PBS and precipitated with 0.5 ml 10 % trichloroacetic acid for 20 min at 4 C (Wang *et al.*, 1996a). The precipitate was washed in methanol twice and solubilized in 0.5 ml 1 N sodium hydroxide. The incorporated radioactivity was measured in a 1217

Rackbeta liquid scintillation counter (LKB Wallac, Turku, Finland). Each experiment was repeated three times.

10. DNA fluorometric assay

In addition to the [^{3}H]thymidine incorporation assay, the effect of E2 on the growth of IOSE-29 and IOSE-29EC was determined by measuring the DNA content as previously described with some modifications in 24-well plates (Rago *et al.*, 1990). The cells were treated with various concentrations (10^{-8}, 10^{-7} or 10^{-6} M) of E2 and/or tamoxifen (10^{-6} M) in phenol-red free medium 199 with charcoal/dextran treated FBS for 6 days. After treatment, the cells were washed with TNE buffer (10 mM Tris, 1 mM EDTA, 2 M NaCl, pH 7.4) three times and stored at –70 C. On the day of assay, 250 µl of distilled water was added in the wells and incubated for 1 h at room temperature (RT). The plates were frozen then for 1 h at –70C and thawed until reaching RT. The amount of DNA was measured using an automated microplate fluorescence reader (Model FL600, Bio-Tek Instruments Inc., Winooski, VA) at an excitation wavelength of 350 nm and emission wavelength of 460 nm (sensitivity = 90) following adding the DNA fluorescent dye. The amount of DNA in the culture was calculated by inserting the fluorescence unit into a standard curve.

11. Quantification of apoptotic cells

To quantify the induction of apoptosis, DNA fragmentation was measured using the cell death detection ELISA kit (Roche Molecular Biochemicals) as previously described (4). Briefly, 1×10^4 cells were plated in each well of 24-well plates. After treatment with E2 and tamoxifen for 6 days, the conditioned media were collected and stored. The cells were washed with PBS, and 0.1

ml lysis buffer was added. Following 15 min incubation on ice, apoptotic cells in cell lysates and conditioned media were assayed for DNA fragments according to the manufacturer's protocol. The same amount (1μg) of cell lysate was used in each experiment. DNA fragmentation was measured at 405 nm against the untreated control.

12. *In vitro* MAPK assay

IOSE-29 and IOSE-29EC cells were serum starved for 4 h. The cells were then treated with FSH (100 ng/ml) in the presence or absence of PD98059 for 10 and 30 min, respectively and washed twice with ice-cold PBS and lysed in 1 x lysis buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, 1 mM PMSF). The extracts were placed on ice for 15 min and centrifuged to remove cellular debris, and protein amount of supernatants was determined. Cellular protein (200 μg) was immunoprecipitated with immobilized phospho-p44/42 MAPK monoclonal antibody. *In vitro* MAPK assays were performed using the Elk-1 fusion protein as a substrate for activated MAPK, according to the manufacturer's suggested procedure (New England Biolabs Inc.).

13. RIA for intracellular cAMP

To measure intracellular cAMP, IOSE cell lines and human granulosa luteal cells (2×10^5 cells) were plated onto 35 mm culture dishes and cultured for 4 days. The cells were then preincubated in serum-free medium containing 0.1% BSA and 0.5 mM 3-isobutyl-1-methylxanthine (IBMX, Sigma-Aldrich Corp.) for 30min, and treated with FSH for 0, 5, 10, 20,

or 60 min. Intracellular cAMP levels were measured using a [^{3}H]-cAMP assay system (Amersham Pharmacia Biotech), according to the manufacturer's suggested procedure.

14. Statistical analysis

Data are shown as the means of two or three individual experiments with triplicate samples, and are presented as the mean ± SD. Expression levels of activin/inhibin subunits and type II activin receptors mRNA were analyzed by Student's *t* test using raw data before transformation to percentage and expressed as the percent change from the control value. For a multiple comparison test, data were analyzed by one-way ANOVA (Analysis of Variance) followed by Tukey's multiple comparison or Dunnett's test using raw data before transformation to percentage by the computer software PRISM GraphPad (Ver. 2, GraphPad Software Inc., San Diego, USA). All data were considered significantly different from each other at $P<0.05$ and expressed as the change from the control value in *the Result*.

III. RESULTS

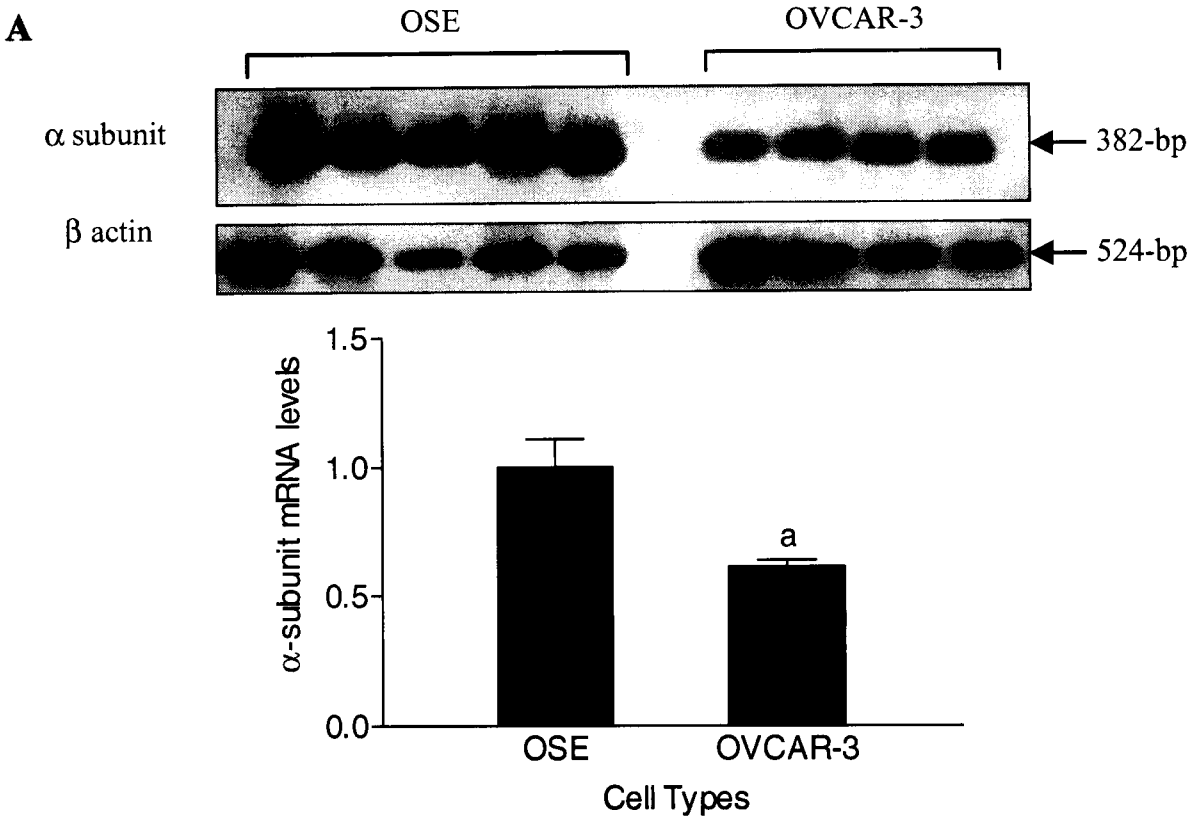
1. *EXPERIMENT A*

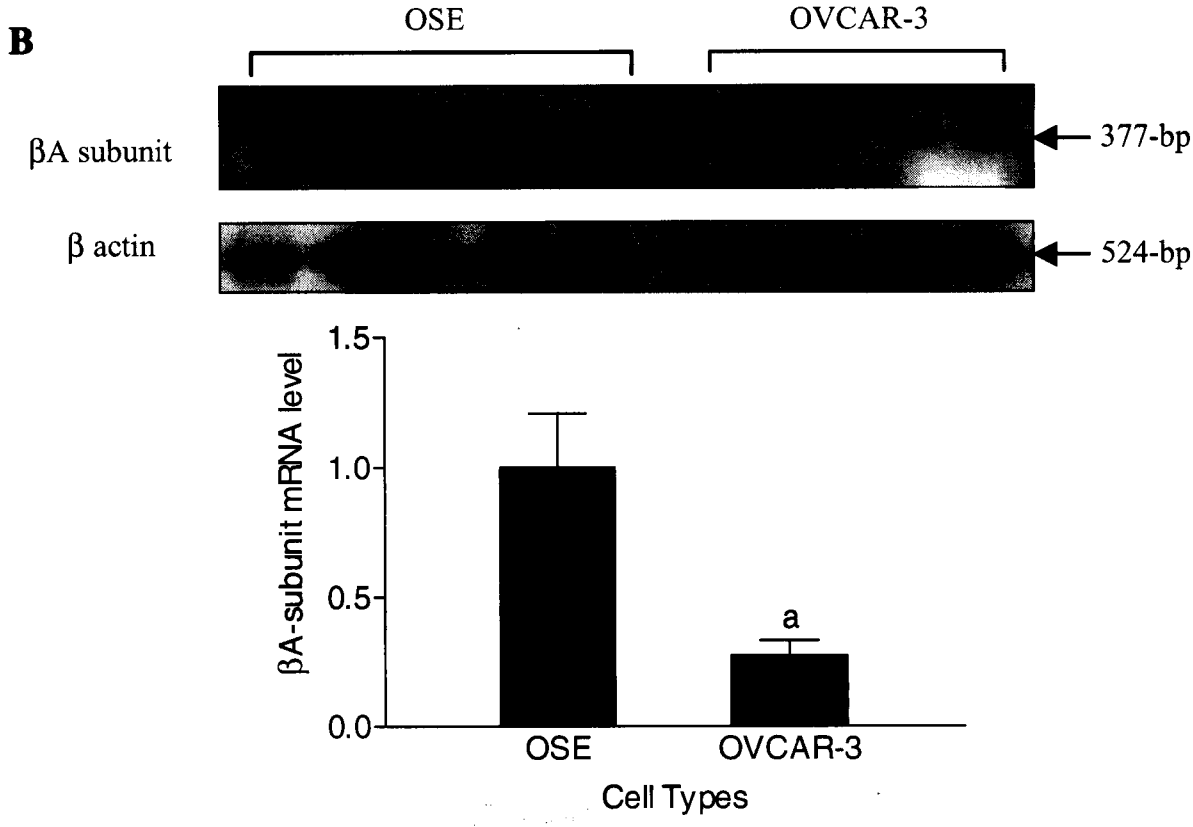
1.1. Expression of activin/inhibin subunits

To investigate the relative importance of activin and inhibin in normal and neoplastic OSE, basal expression levels of α, βA and βB subunits in OSE and OVCAR-3 were examined. The mRNA levels of activin/inhibin subunits were quantitated by RT-PCR. The 382-bp, 377-bp and 424-bp PCR products were obtained and confirmed as α, βA and βB subunits of activin/inhibin using Southern blot hybridization, respectively, as previously described (Fukuda *et al.*, 1998). The PCR products amplified were subcloned and sequenced, and found to be 100% identical to published sequences of activin/inhibin subunits in these cells (data not shown). As shown in Fig. 8, a significantly higher expression level of α subunit was observed in normal OSE cells when compared to OVCAR-3 cells (Fig. 8A). Also, βA subunit was significantly higher in normal OSE, when compared to OVCAR-3 cells (Fig. 8B). In contrast, a significantly higher mRNA level of βB subunit was detected in OVCAR-3 cells when compared to normal OSE cells (Fig. 8C).

1.2. Expression of activin receptors

Basal expression levels of activin type II receptors (IIA and IIB) in normal and neoplastic OSE cells were investigated. The 456-bp and 699-bp PCR products were obtained and confirmed as activin receptor IIA and IIB using Southern blot hybridization, respectively, as previously described (Fukuda *et al.*, 1998). The PCR products amplified were subcloned and sequenced, and found to be 100% identical to published sequences of activin type IIA and IIB receptors in


A
OSE
OVCAR-3
α subunit
β actin
382-bp
524-bp
α-subunit mRNA levels
1.5
1.0
0.5
0.0
a
OSE
OVCAR-3
Cell Types


B
OSE
OVCAR-3
βA subunit
β actin
377-bp
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1.5
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a
OSE
OVCAR-3
Cell Types

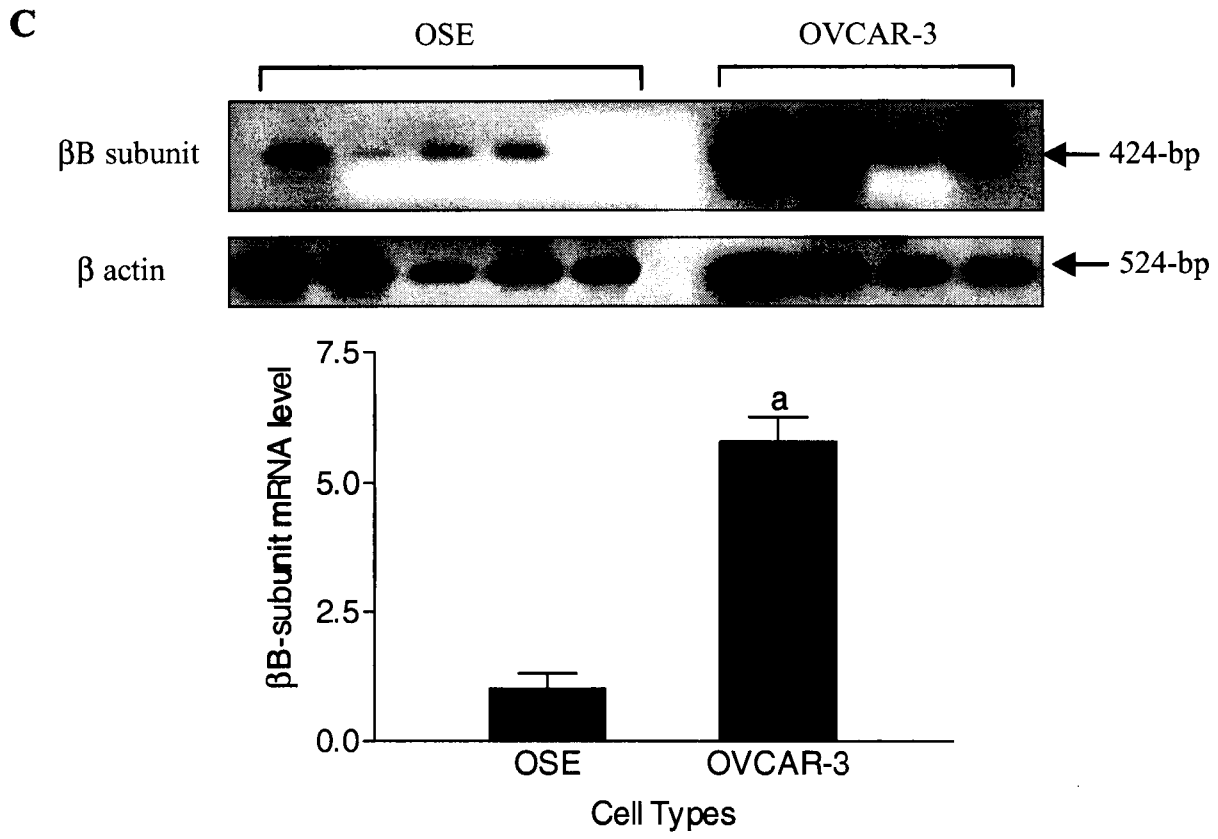


Figure 8. Differential expression level of activin/inhibin subunits in normal OSE and OVCAR-3 cells. The mRNA levels of α (A), βA (B) and βB (C) subunits were quantitated by RT-PCR as described in the *Materials and Methods*. The 382-bp, 377-bp and 424-bp PCR products were obtained and confirmed as α, βA and βB subunits of activin/inhibin using Southern blot hybridization, respectively. Values are standardized with β-actin expression and represented as the mean ± SD. a, $P<0.05$ *vs* basal expression level of activin/inhibin subunits in OSE.

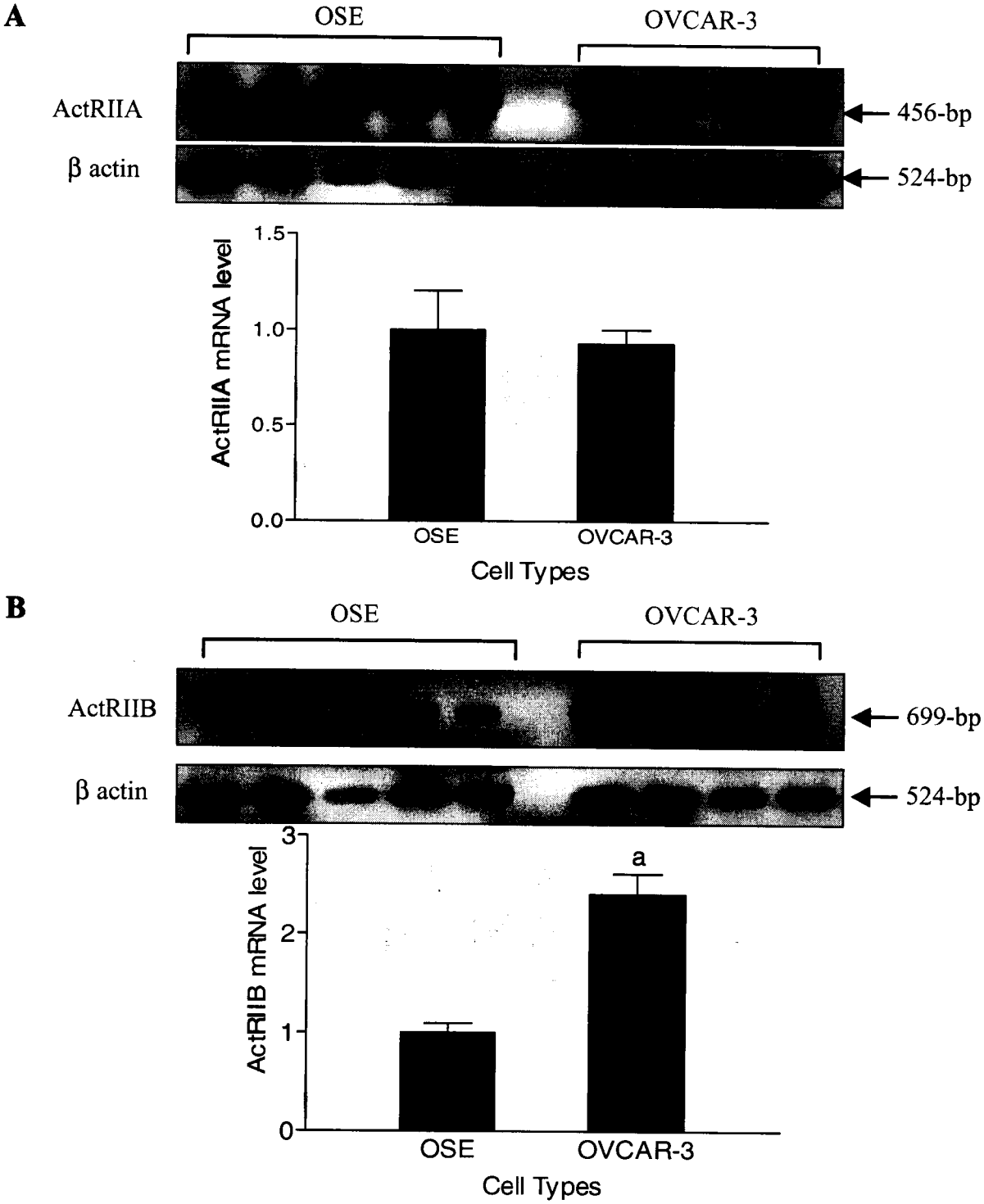


Figure 9. Differential expression level of type II activin receptors in normal OSE and OVCAR-3 cells. The mRNA levels of activin receptor IIA (A) and IIB (B) were quantitated by RT-PCR. The 456-bp and 699-bp PCR products were obtained and confirmed as activin receptor IIA and IIB using Southern blot hybridization, respectively. Values are standardized with β-actin expression and represented as the mean ± SD. a, $P<0.05$ *vs* basal expression level of each activin receptor in OSE.

these cells (data not shown). As seen in Fig 9A, no difference of activin receptor IIA was observed in normal OSE and OVCAR-3 cells. Interestingly, a significantly higher level of activin receptor IIB mRNA was observed in OVCAR-3 cells when compared to normal OSE cells (Fig. 9B).

1.3. Effects of activin on cell proliferation

The effect of activin on cellular proliferation was analyzed in normal and neoplastic OSE cells using a thymidine incorporation assay. As shown in Fig. 10, rh-activin A (1, 10, 8and 100 ng/ml) induced a significant increase in cell proliferation to 150 % of control in OVCAR-3 cells. This stimulatory effect of activin was attenuated by treatment of follistatin (100 ng/ml), which is a specific activin binding protein. However, no difference was observed following activin treatment in normal OSE cells (data not shown).

1.4. Effects of activin on activin/inhibin subunits and activin receptors

To investigate whether the growth stimulatory effect of activin in neoplastic OSE is mediated via an autocrine mechanism, the effect of exogenous activin on the expression of activin/inhibin subunits and activin receptors in OVCAR-3 cells were investigated. Treatment of rh-activin A at concentrations of 1, 10, and 100 ng/ml for 24 h induced a significant increase in α subunit mRNA expression (Fig. 11). Interestingly, a dose-dependent increase in βA subunit mRNA was observed after activin treatments (Fig. 12A). In addition, this stimulatory effect of activin (10 ng/ml) on βA subunit mRNA expression was attenuated with treatment of follistatin (Fig. 12B). However, no difference in βB subunit was observed in OVCAR-3 cells following rh-activin A

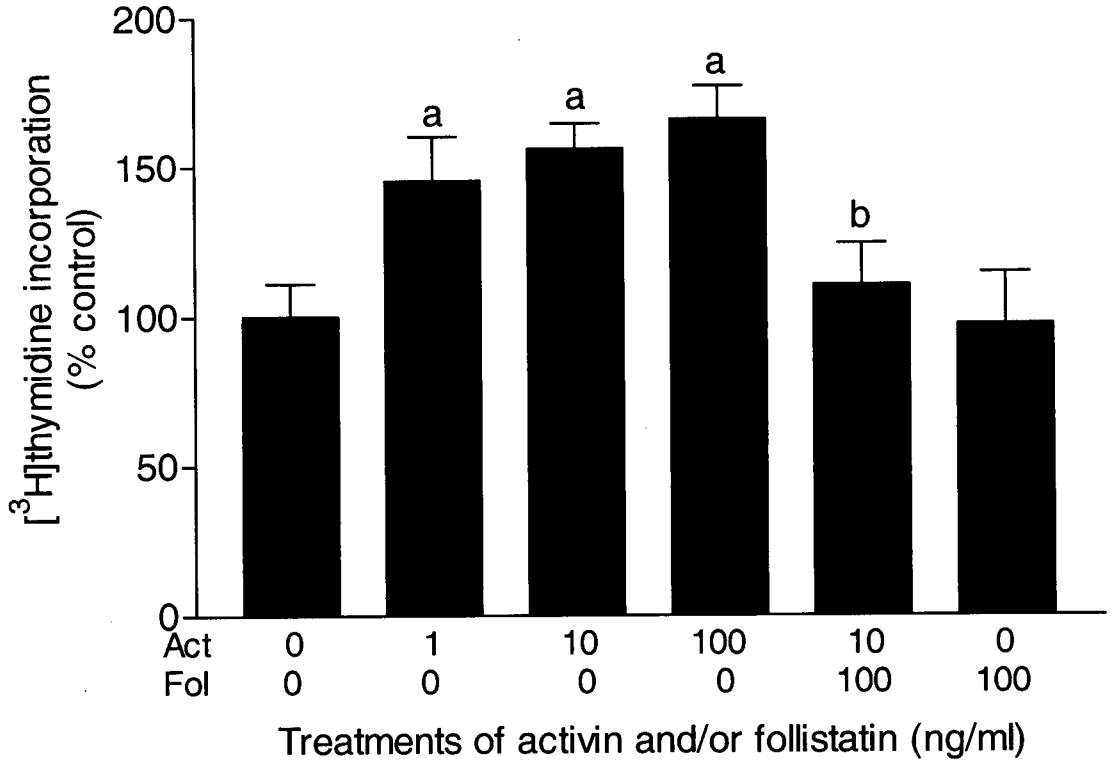


Figure 10. Effects of rh-activin A on OVCAR-3 cell proliferation. Normal OSE and OVCAR-3 cells were cultured and treated with rh-activin A (1, 10, and 100 ng/ml) and/or follistatin (100 ng/ml) as described in the *Materials and Methods*. A [^{3}H]thymidine incorporation assay was performed to quantify DNA synthesis following activin treatments. Values are the mean ± SD for three individual experiments, each with triplicate samples. a, $P<0.05$ *vs* untreated control; b, $P<0.05$ *vs* 10 ng/ml activin treatment.

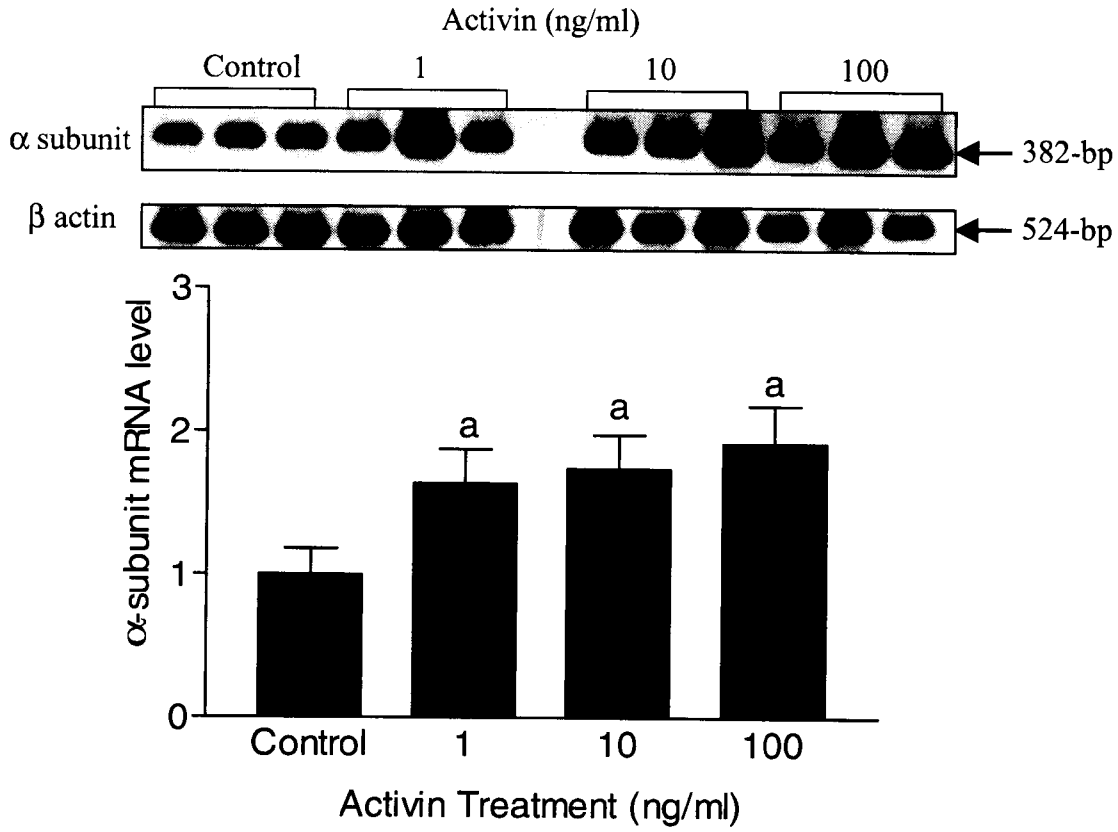


Figure 11. Effect of rh-activin A on α subunit expression in OVCAR-3 cells. Cells were treated with activin (1, 10 and 100 ng/ml) for 24 h as described in the *Materials and Methods*. To investigate the specificity of activin, the cells were treated with activin (10 ng/ml) plus follistatin (100 ng/ml) simultaneously. The expected size of PCR product for α subunit was obtained as 382-bp. Values are standardized with β-actin expression and represented as the mean ± SD for three individual experiments, each with triplicate samples. a, $P<0.05$ *vs* untreated control.

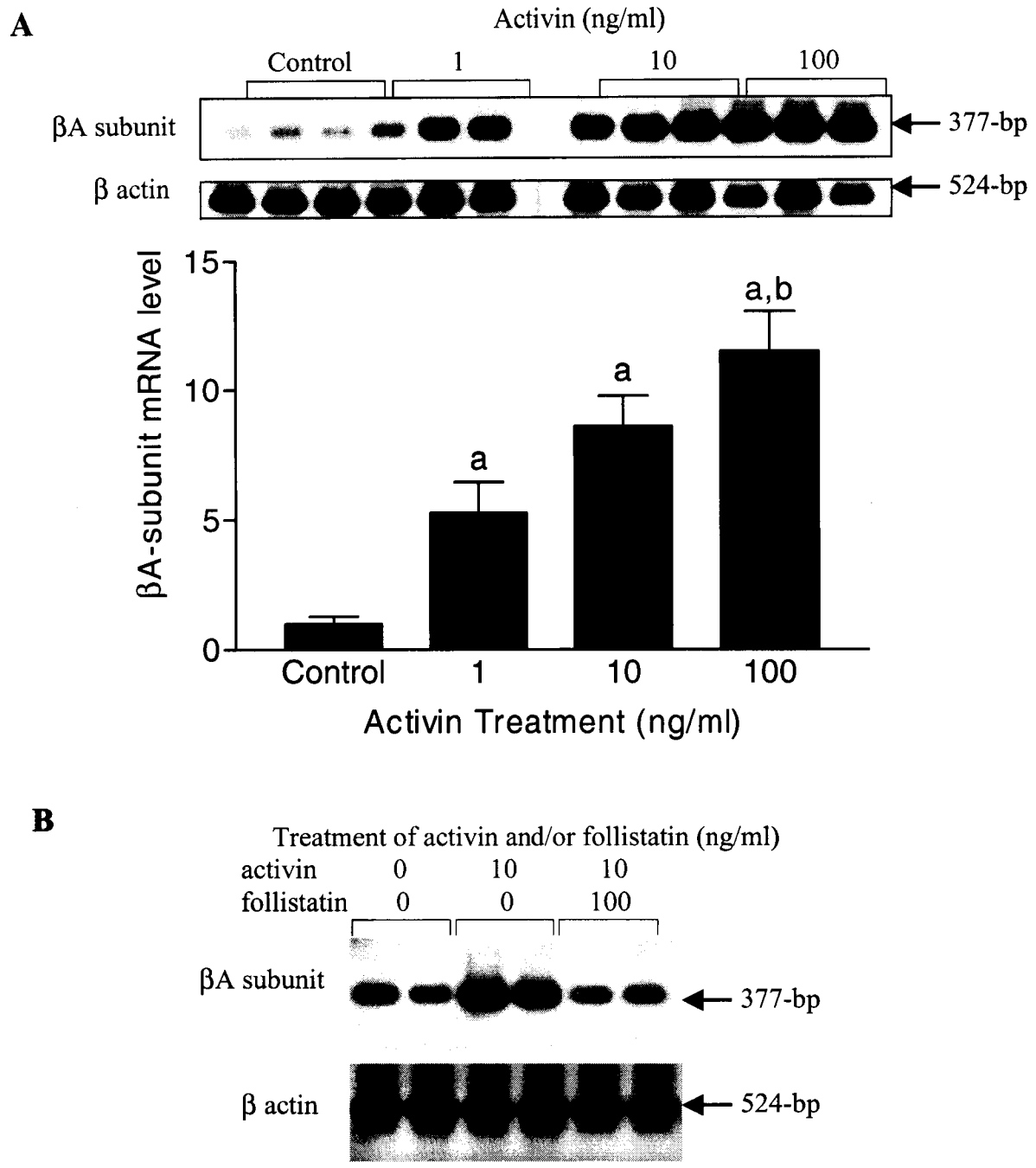


Figure 12. Effect of rh-activin A on βA subunit expression in OVCAR-3 cells. Cells were treated with activin (1, 10 and 100 ng/ml) for 24 h as described in the *Materials and Methods* (A). To investigate the specificity of activin, the cells were treated with activin (10 ng/ml) plus follistatin (100 ng/ml) simultaneously (B). The expected size of PCR product for βA was obtained as 377-bp. Values are standardized with β-actin expression and represented as the mean ± SD for three individual experiments, each with triplicate samples. a, $P<0.05$ *vs* untreated control; b, $P<0.05$ vs 1 ng/ml activin treatment.

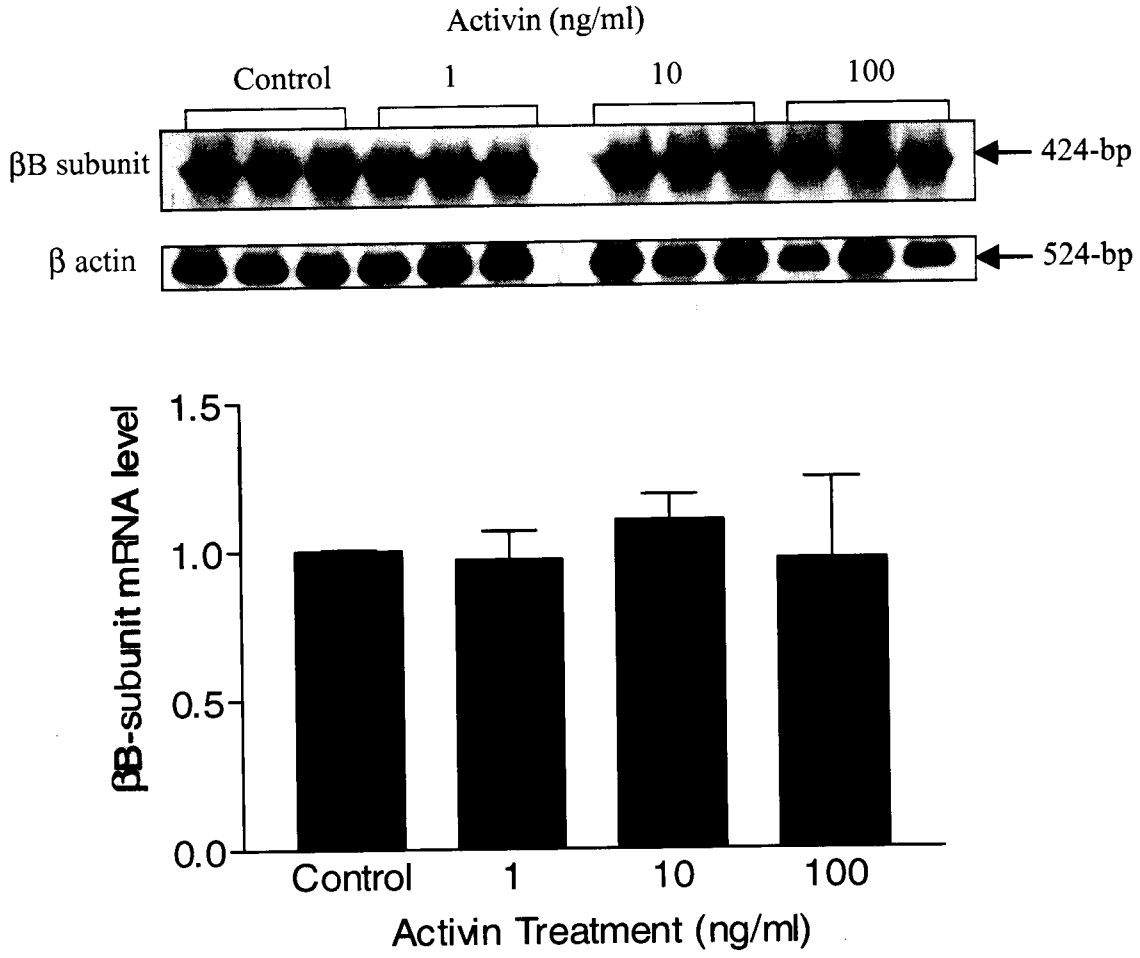


Figure 13. Effect of rh-activin A on βB subunit expression in OVCAR-3 cells. Cells were treated with activin (1, 10 and 100 ng/ml) for 24 h as described in the *Materials and Methods*. To investigate the specificity of activin, the cells were treated with activin (10 ng/ml) plus follistatin (100 ng/ml) simultaneously. The expected size of PCR product for βB was obtained as 424-bp. Values are standardized with β-actin expression and represented as the mean ± SD for three individual experiments, each with triplicate samples.

treatments (Fig. 13). Further, the levels of activin receptor IIA and IIB were determined after rh-activin A treatments. Treatments of OVCAR-3 cells with activin (1 - 100 ng/ml) did not affect activin receptor IIA and IIB mRNA levels in OVCAR-3 cells (Fig. 14A and B).

The effect of activin on activin/inhibin α and βA subunits was further investigated in a time-course experiment (Fig. 15A and B). OVCAR-3 cells were treated with 10 ng/ml activin A for 3, 6, 12 and 24 h. Treatment of activin (10 ng/ml) for 24 h induced a significant increase in α subunit mRNA level as shown in Fig. 15A. The βA subunit mRNA level also significantly increased following activin treatment at same amount for 6, 12 and 24 h (Fig. 15B). Thus, exogenous rh-activin A increased the mRNA levels of α and βA subunit in a time-dependent manner.

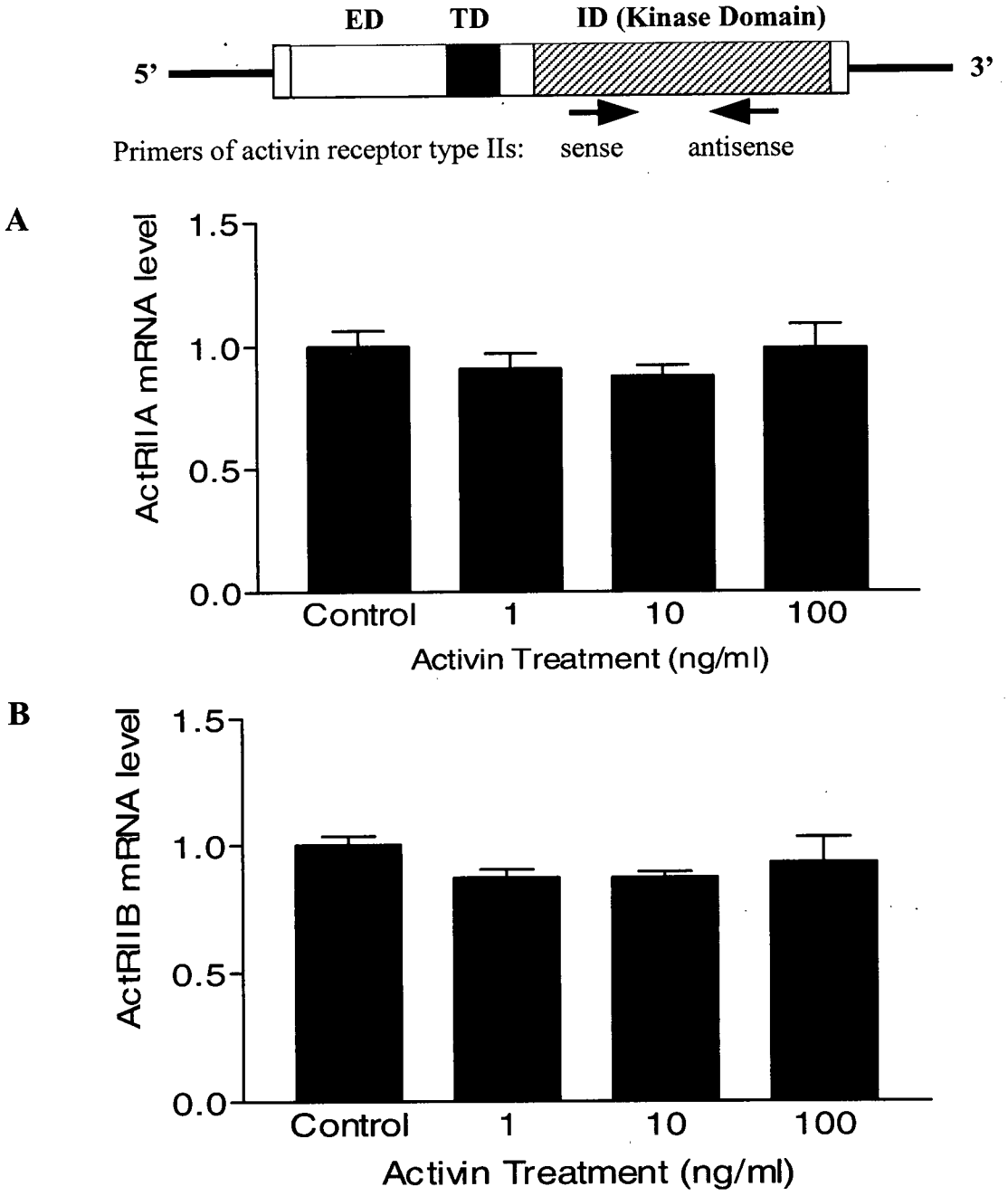


Figure 14. Effects of rh-activin A on type II activin receptors mRNA expression in OVCAR-3 cells. Cells were treated with 1, 10 and 100 ng/ml activin for 24 h as described in the *Materials and Methods*. Primer sets for activin receptor IIA and IIB were designed to amplify coding sequences of intracellular domain of type II activin receptors. The 456-bp and 699-bp of PCR products were observed for activin type IIA (A) and IIB (B) receptors as previously published. Values are standardized with β-actin expression and represented as the mean ± SD for two individual experiments, each with triplicate samples. ED, extracellular domain; TD, transmembrane domain; ID, intracellular domain of activin receptors.

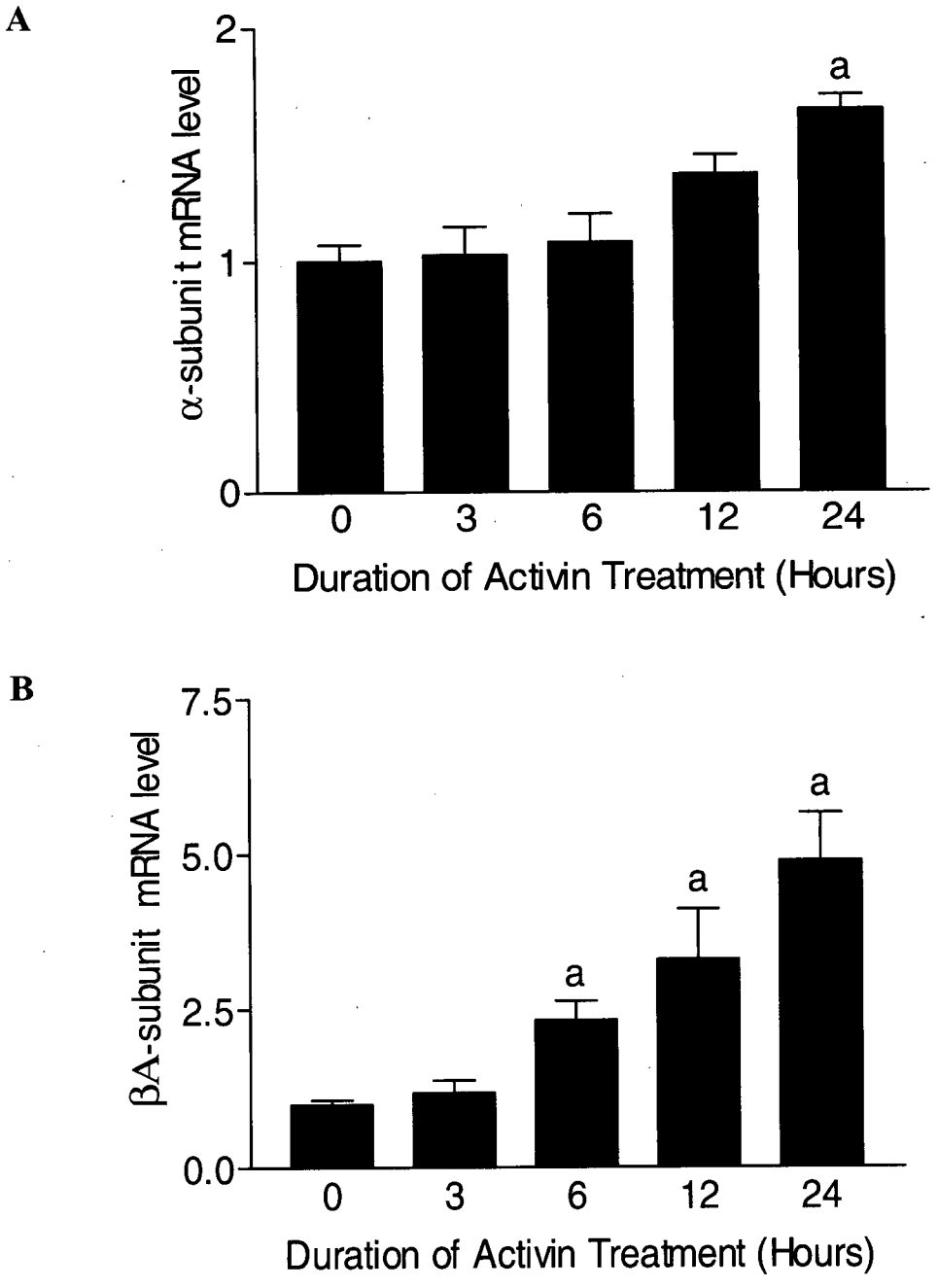


Figure 15. A time-dependent increase of α and βB subunit by rh-activin A in OVCAR-3. The effect of activin on activin/inhibin α (A) and βA (B) subunits was further investigated in a time-course experiment. The cells were treated with 10 ng/ml activin for 3, 6, 12 and 24 h as described in the *Materials and Methods*. Values are relative to β-actin and plotted as the mean ± SD for three individual experiments, each with duplicate samples. a, $P<0.05$ *vs* untreated control.

2. *EXPERIMENT B*

2.1. Expression of activin/inhibin subunit mRNAs

The mRNA levels of α, βA, and βB subunits in IOSE-29 (passages 13-16), IOSE-29EC (passages 15-17), IOSE-29EC/T4 (T4), and IOSE-29EC/T5 (T5) were investigated by RT-PCR and Southern blot analysis. The possibility of cross-contamination was rule out, because no PCR products were observed and detected in the negative control [TmA(-); without template in the PCR reaction] by ethidium bromide (data not shown) and Southern blot analysis (Fig. 16). Linear relationship between PCR products and amplification cycles was obtained in GAPDH, α, βA and βB subunits in all cell types (data not shown). Expected PCR products of GAPDH, α, βA and βB subunits were obtained at 373-bp, 382-bp, 377-bp and 424-bp respectively and confirmed by Southern blot analysis (Fig. 16) and sequence analysis (data not shown). Semi-quantitative analysis of the present study demonstrated that all types of activin/inhibin subunits are expressed in IOSE-29, IOSE-29EC, T4, and T5. Interestingly, βB subunit was less expressed in IOSE cell lines when compared in OVCAR-3 cells (Fig. 16).

2.2. Expression of activin receptor mRNAs

The mRNA levels of activin receptor IA, IB, IIA, and IIB in IOSE-29 (passages 13-16), IOSE-29EC (passages 15-17), T4 and T5 were investigated by RT-PCR and Southern blot analysis (Fig. 17). The expected sizes of PCR products for activin receptors were obtained as 651-bp, 684-bp, 456-bp and 699-bp, respectively using sense and antisense primers located within the intracellular kinase domains of each activin receptor. The PCR of GAPDH was amplified to rule out the possibility of RNA degradation, and used to control the variation in mRNA concentrations in RT reaction. PCR products of the predicted sizes were obtained and

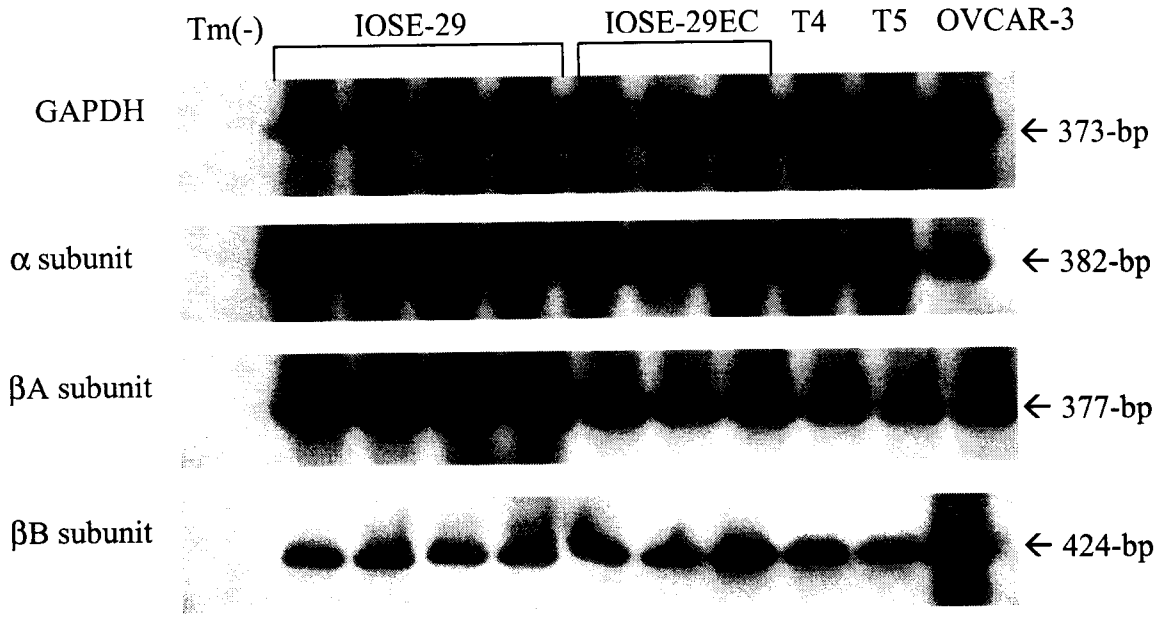


Figure 16. The mRNA expressions of activin/inhibin subunits in IOSE cell lines. The mRNA levels of α, βA, and βB subunits in IOSE-29 (passages 13-16), IOSE-29EC (passages 15-17), IOSE-29EC/T4 (T4), and IOSE-29ECT5 (T5) were investigated by RT-PCR and Southern blot analysis as previously described in the *Materials and Methods*. The possibility of cross-contamination was ruled out, because no PCR products were observed and detected in the negative control [TmA(-); without template in the PCR reaction] by ethidium bromide (data not shown) and Southern blot analysis. The expected sizes of PCR product for GAPDH, α, βA and βB subunits were obtained as 373-bp, 382-bp, 377-bp and 424-bp, respectively.

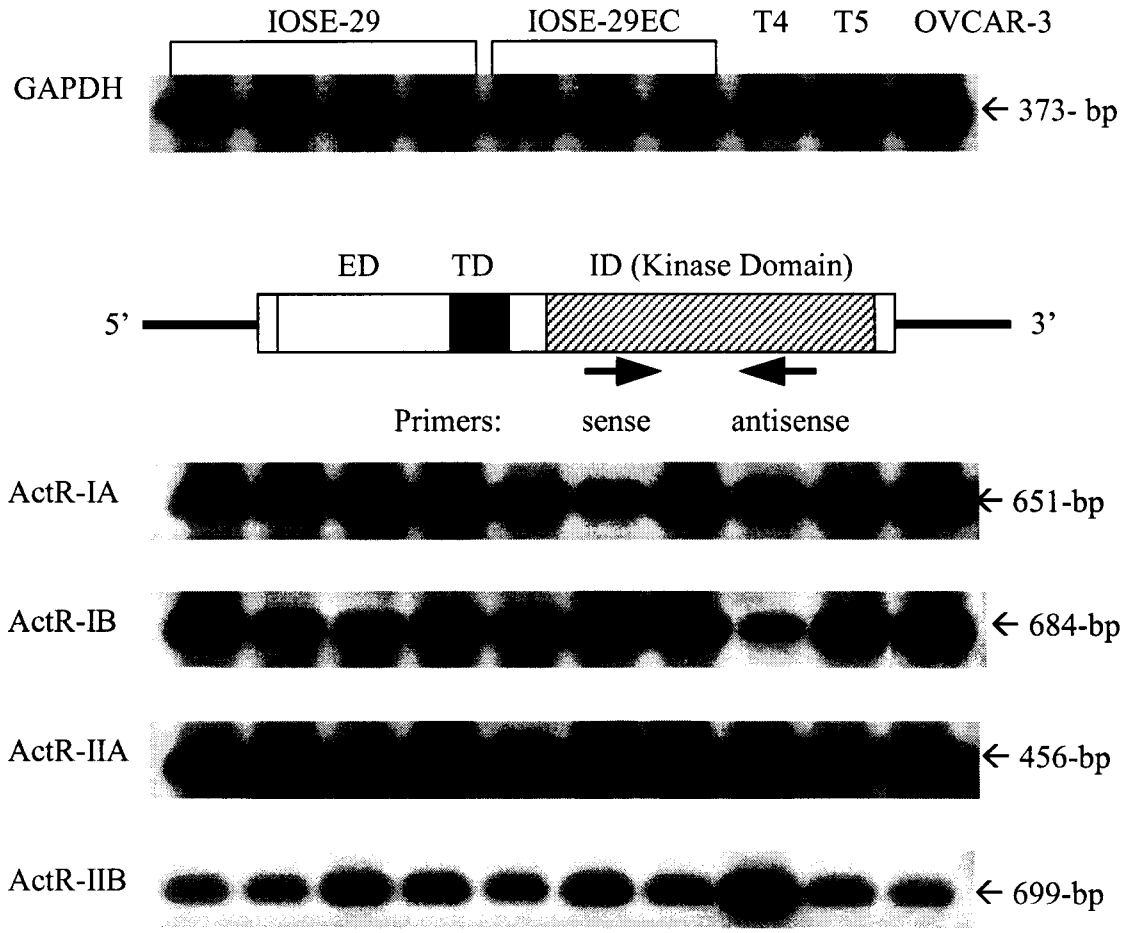


Figure 17. The mRNA expressions of activin receptors in IOSE cell lines. The mRNA levels of activin receptor IA, IB, IIA, and IIB in IOSE-29 (passages 13-16), IOSE-29EC (passages 15-17), T4, and T5 were investigated by RT-PCR and Southern blot analysis. The expected sizes of PCR products for activin receptors were obtained as 651-bp, 684-bp, 456-bp and 699-bp, respectively using sense and antisense primers located within the intracellular kinase domains of activin receptors. The PCR of GAPDH was amplified to rule out the possibility of RNA degradation, and used to control the variation in mRNA concentrations in RT reaction. ED, extracellular domain; TD, transmembrane domain; ID, intracellular domain of activin receptors.

confirmed by Southern blot analysis using DIG-labeled probes (Fig. 17) and sequence analysis (data not shown). Semi-quantitative analysis of the present study demonstrated that all forms of activin receptors were observed in IOSE-29, IOSE-29EC, T4 and T5.

2.3. Expression of activin receptor proteins

Immunoblot analysis was performed using the rabbit polyclonal antibodies against activin receptor IA (amino acid 474-494), IB (aa 493-505), IIA (aa 482-494), and IIB (aa 524-536) based on COOH terminal amino acids in IOSE cell lines. As shown in Fig. 18, activin receptor IA protein (60 kDa) was observed in all cell types. OVCAR-3 cell line was used for positive control of the expression of activin receptors (Fukuda *et al.*, 1998). Similarly, activin receptor IB protein (55 kDa) was also observed in all cell types (Fig. 18). In addition, activin receptor IIA and IIB were clearly detected at 80 kDa and 60 kDa respectively in IOSE cell lines and OVCAR-3 cells (Fig. 18). Immunoblot analysis of the present study demonstrated that all forms of activin receptor protein were observed in IOSE-29, IOSE-29EC, T4 and T5.

2.4. Effects of activin on cell number

To evaluate the role of recombinant human activin A (rh-activin A) in IOSE cell lines, IOSE-29 and IOSE29EC were treated with different concentrations (1, 10, and 100 ng/ml) of rh-activin A for 6 days. The proliferative index was measured by thymidine incorporation assay. Follistatin, which is activin-binding protein, was used to block the action of activin in cell proliferation study. As seen in Fig. 19, a dose-dependent decrease of cell number was observed following rh-activin A (1, 10, and 100 ng/ml) treatments in IOSE-29 (Fig. 19A, 100.0 ± 7.63 *vs* 83.7 ± 6.06, 67.9 ± 4.10 or 59.9 ± 9.06) and IOSE-29EC (Fig. 19B, 100.0 ± 5.89 *vs* 75.9 ± 9.11,61.4 ± 8.11 or 52.9 ± 9.70) cells. Co-treatment with follistatin (100ng/ml) with activin blocked the growth-

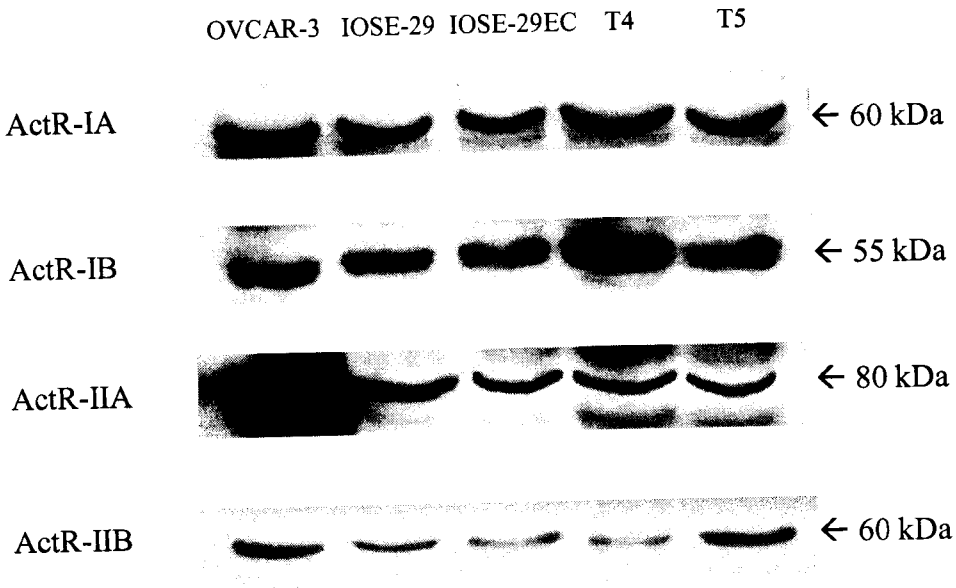


Figure 18. The expression of activin receptor proteins in IOSE cell lines. Immunoblot analysis was performed using the rabbit polyclonal antibodies against activin receptor IA, IB, IIA and IIB as previously described in the *Materials and Methods.* Activin receptor IA (60 kDa) and IB (55 kDa) were observed in all cell types. OVCAR-3 cell line was used for positive control of the expression of activin receptors (Fukuda *et al.*, 1998). In addition, activin receptor IIA and IIB were clearly detected at 80 kDa and 60 kDa, respectively, in IOSE cell lines and OVCAR-3 cells.

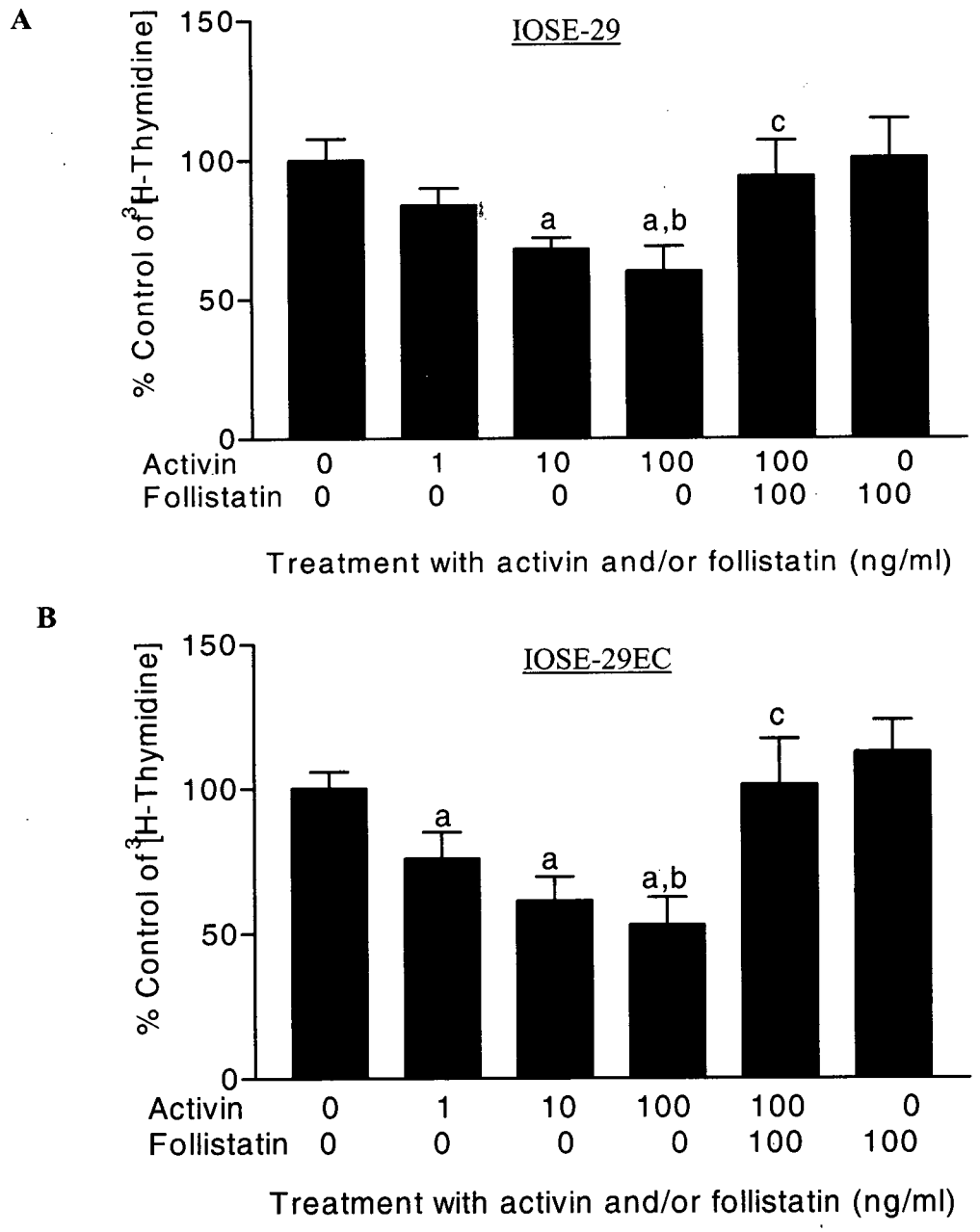


Figure 19. Effect of activin on cell proliferation in IOSE cell lines. The IOSE-29 and IOSE-29EC were treated with different concentrations (1, 10, and 100 ng/ml) of rh-activin A for 6 days. Proliferative index was measured using the thymidine incorporation assay as previously described in the *Materials and Methods*. A dose-dependent decrease was observed following rh-activin A (1, 10, and 100 ng/ml) treatments in IOSE-29 (A) and IOSE-29EC (B). Co-treatment with follistatin (100ng/ml) with activin was demonstrated to block the growth-inhibitory of activin in both cell lines. Values are the mean ± SD for three individual experiments, each with triplicate samples. a, $P<0.05$ *vs* untreated control; b, $P<0.05$ *vs* 1 ng/ml activin treatment; c, $P<0.05$ *vs* treatment with 100 ng/ml activin.

inhibitory effect of activin in both cell lines (Fig. 19). However, no significant difference was observed in follistatin treatment only.

2.5. Effects of TGF-β on cell proliferation

To examine the role of TGF-β, normal OSE, IOSE-29 and IOSE-29EC cells were treated with different concentrations (0.1, 1 and 10 ng/ml) of TGF-β for 72 h. As seen in Fig. 20A, TGF-β (1 and 10 ng/ml) induced a significant decrease of normal OSE cell proliferation in a dose-dependent manner (100.0 ± 15.62 *vs* 58.6 ± 11.78 or 43.3 ± 12.03). Also, a significant decrease was observed following TGF-β treatments (1-10 ng/ml) in IOSE-29 (Fig. 20B, 100.0 ± 5.03 *vs* 81.1 ± 7.59 or 69.8 ± 4.08). In addition, treatment with TGF-β resulted in a decrease of proliferative index in IOSE-29EC (Fig. 20C, 100.0 ± 11.70 *vs* 74.2 ± 5.63, 67.1 ± 7.05 or 55.0 ± 6.75).

2.6. Effects of activin and TGF-β on apoptosis

To quantify the induction of apoptosis, IOSE-29EC cells were treated with rh-activin A for 6 days. As shown in Fig 21, treatments with 10 and 100 ng/ml activin increased DNA fragmentation in a dose-dependent manner (100.0 ± 8.06 *vs* 190.6 ± 13.58 or 221.3 ± 15.72). Co-treatment with follistatin (100ng/ml) with activin attenuated the effect of activin. No significant difference was observed in follistatin treatment only in IOSE-29EC cells. Similarly, IOSE-29EC cells were treated with different concentrations of TGF-β for 72 h. Treatments with TGF-β induced a significant increase of DNA fragmentation in a dose-dependent manner in IOSE-29EC (Fig. 22, 100.0 ± 5.20 *vs* 123.7 ± 10.03, 191.3 ± 16.94 or 201.9 ± 25.06).

A

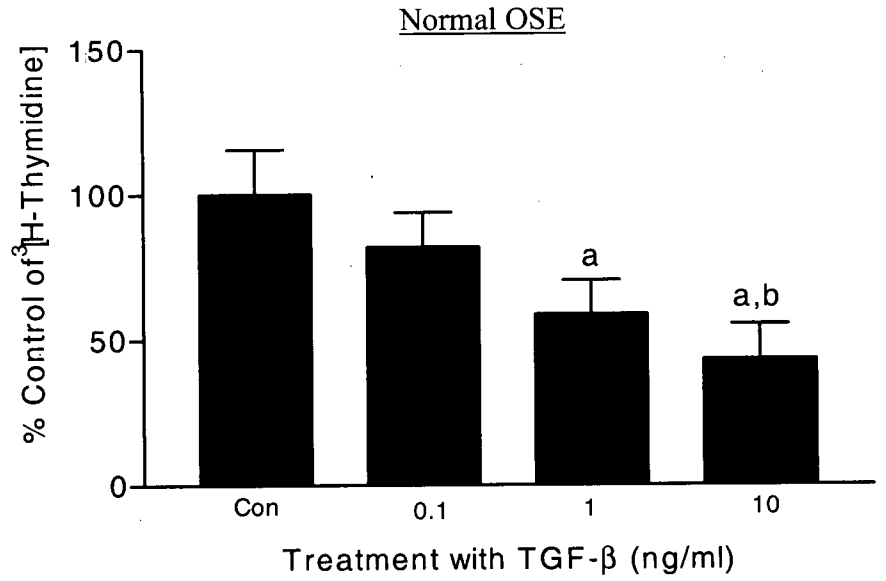
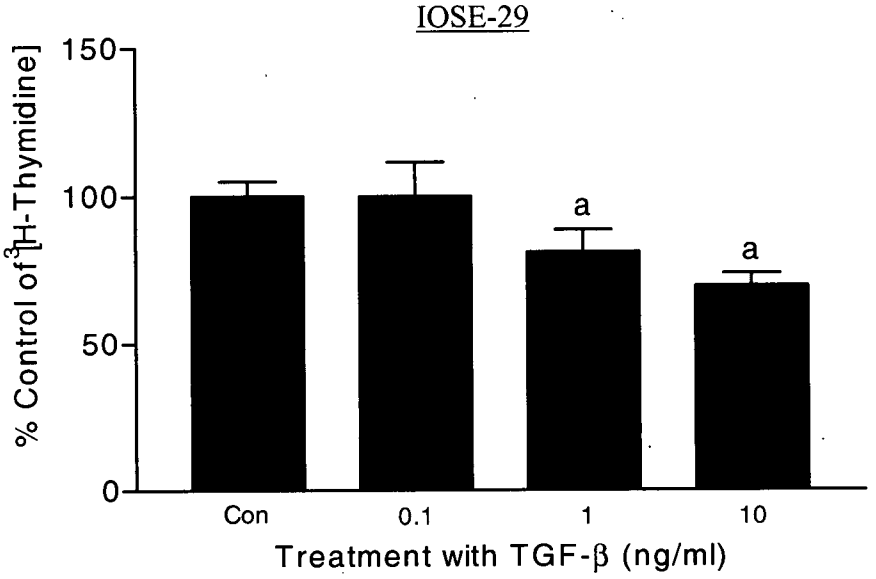
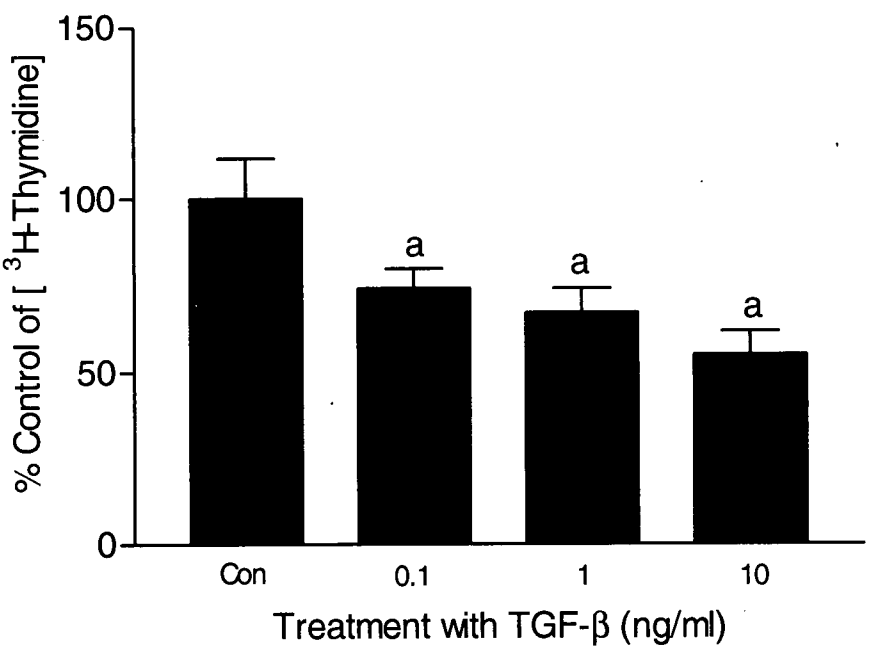


Figure 20 (including next page). Effects of TGF-β on cell proliferation in IOSE cell lines. Normal OSE, IOSE-29 and IOSE-29EC cells were treated with increasing concentrations (0.1, 1 and 10 ng/ml) of TGF-β for 72 h. Proliferative index was measured using the thymidine incorporation assay as previously described in the *Materials and Methods*. Treatments with TGF-β (1 and 10 ng/ml) induced a significant decrease of growth in normal OSE (A), IOSE-29 (B) and IOSE-29EC (C) in a dose-dependent manner. Values are the mean ± SD for three individual experiments, each with triplicate samples. a, $P<0.05$ *vs* untreated control; b, $P<0.05$ *vs* 0.1 ng/ml TGF-β treatment.

B


IOSE-29
% Control of [3H-Thymidine]
150
100
50
0
a
a
Con
0.1
1
10
Treatment with TGF-β (ng/ml)

C


IOSE-29EC
% Control of [3H-Thymidine]
150
100
50
0
a
a
a
Con
0.1
1
10
Treatment with TGF-β (ng/ml)

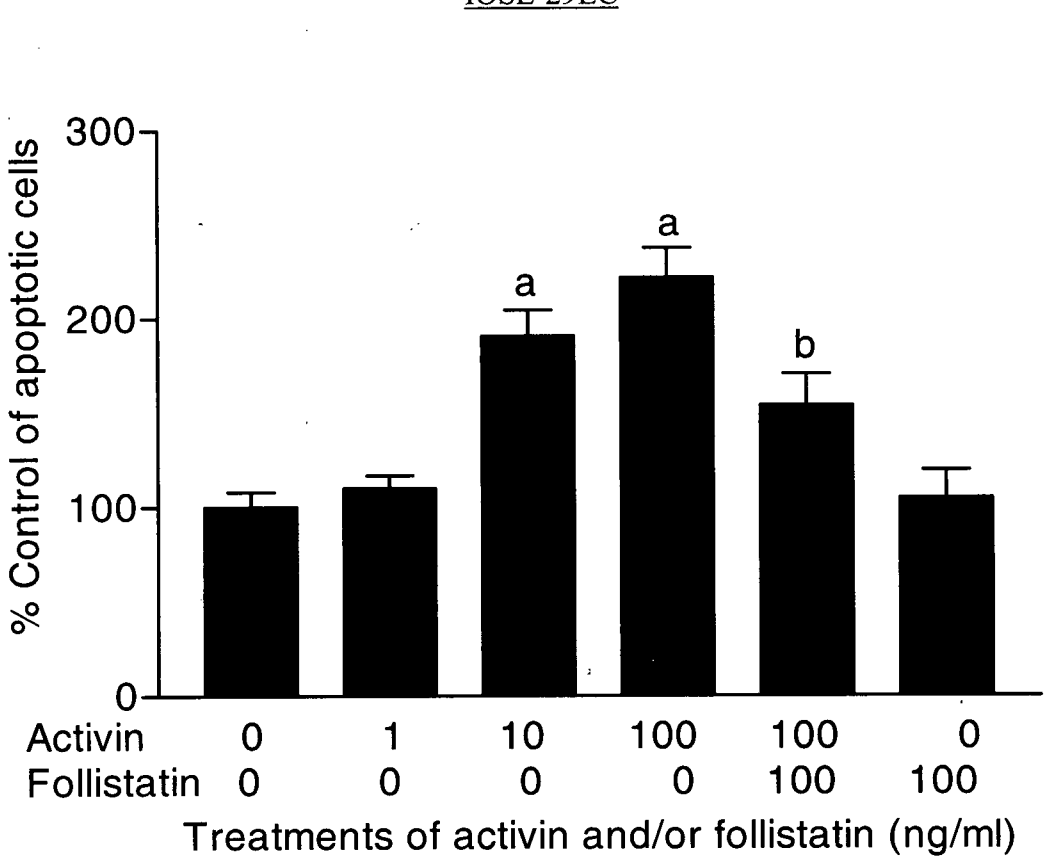


Figure 21. Effect of activin in the induction of apoptosis. To quantify the induction of apoptosis, IOSE-29EC cells were treated with rh-activin A for 6 days. Attached and detached cells were collected and DNA fragmentation was measured by cell death detection ELISA as described in the *Material and Methods*. Treatments with 10 and 100 ng/ml activin increased DNA fragmentation in a dose-dependent manner. Co-treatment with follistatin (100ng/ml) with activin attenuated the effect of activin. Values are the mean ± SD for two individual experiments, each with triplicate samples. a, $P<0.05$ *vs* untreated control; b, $P<0.05$ *vs* treatment with 100 ng/ml activin.

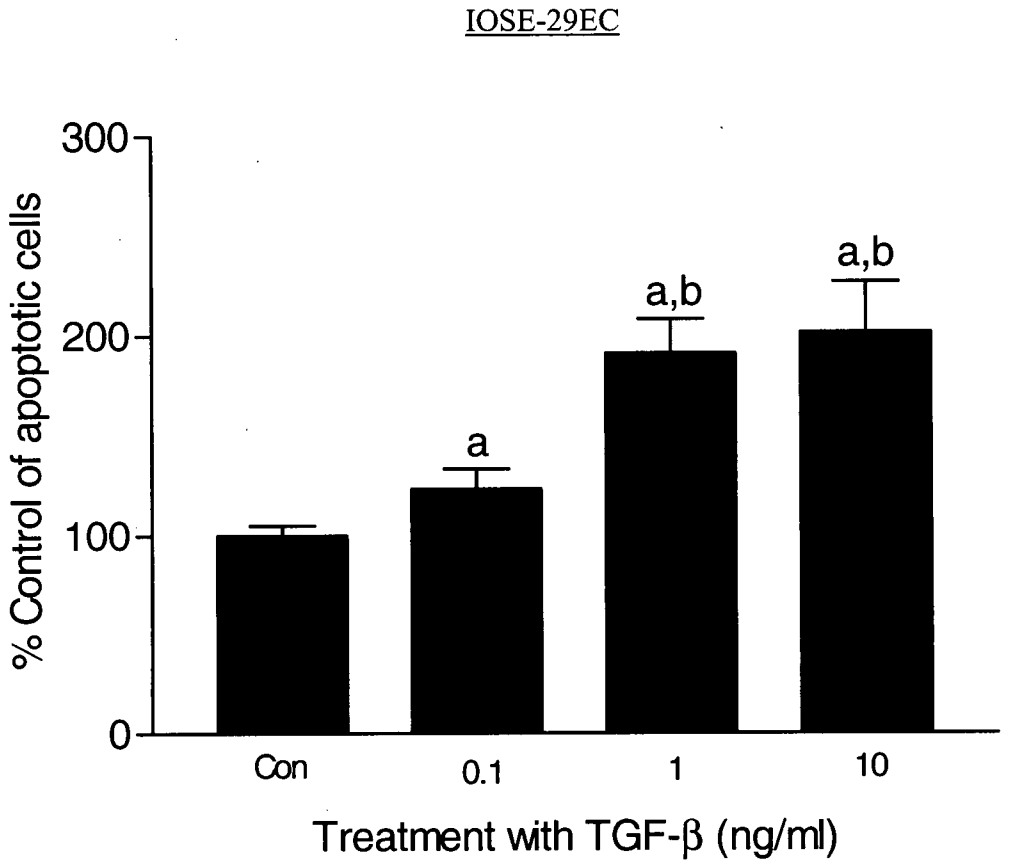


Figure 22. Effect of TGF-β in the induction of apoptosis. To quantify the induction of apoptosis, IOSE-29EC cells were treated with TGF-β for 72 h. Attached and detached cells were collected and DNA fragmentation was measured by cell death detection ELISA as described in the *Material and Methods*. Treatments with TGF-β for 72 h induced DNA fragmentation of IOSE-29EC in a dose-dependent manner. Values are the mean ± SD for two individual experiments, each with triplicate samples. a, $P<0.05$ *vs* untreated control; b, $P<0.05$ *vs* treatment with 0.1 ng/ml TGF-β treatment.

2.7. Expression of pro- and anti-apoptotic gene mRNAs

The mRNA levels of bax and bcl-2 in IOSE-29 (passages 13-18), IOSE-29EC (passages 13-18) were investigated by RT-PCR and Southern blot analysis (Fig. 23). Linear relationship between PCR products and amplification cycles was obtained in GAPDH, bax and bcl-2 in all cell types (data not shown). PCR products of GAPDH, bax and bcl-2 were obtained as 373-bp, 323-bp and 459bp, respectively and confirmed by Southern blot analysis using DIG-labeled probes (Fig. 23). No difference was observed in the expression level of bax mRNA between IOSE-29 and IOSE-29EC cells. In contrast, the expression level of bcl-2 mRNA was higher in IOSE-29EC cells than IOSE-29 cells (Fig. 23).

2.8. Effects of activin and TGF-β on apoptotic proteins

To investigate the mechanism of activin and TGF-β in the induction of apoptosis, the regulation of apoptotic bax and bcl-2 was examined by immunoblot analysis. The IOSE-29EC cells were treated with increasing doses of rh-activin A and TGF-β respectively for 24 h and immunoblot analysis was performed as described in *the Material and Methods*. Bax and bcl-2 protein were detected at 21 kDa and 26 kDa respectively. As seen in Fig 8A, treatments with 10 and 100 ng/ml activin had no effect on both bax and bcl-2 proteins in these cells. No significant difference of bax protein was observed in TGF-β treatments (Fig 8B). In contrast, treatments with 1 and 10 ng/ml TGF-β induced a significant decrease of bcl-2 protein up to 50% (Fig. 8B and C, 100.0 ± 5.17 *vs* 58.2 ± 7.35 or 54.0 ±5.39). The loaded amount of proteins in treatment groups was normalized by actin protein (41 kDa).

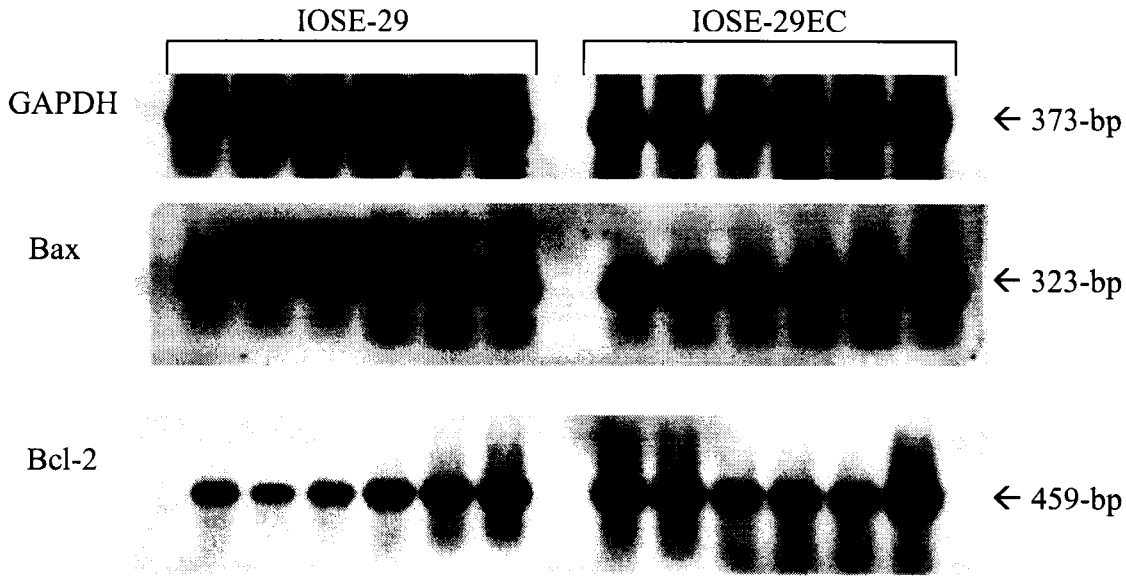


Figure 23. The expression of bax and bcl-2 mRNAs in IOSE cell lines. The mRNA levels of bax and bcl-2 in IOSE-29 (passages 13-18), IOSE-29EC (passages 15-20) were investigated by RT-PCR and Southern blot analysis. PCR products of GAPDH, bax and bcl-2 were obtained as 373-bp, 323-bp and 459-bp, respectively and confirmed by Southern blot analysis using DIG-labeled probes. No difference was observed in the expression level of bax mRNA between IOSE-29 and IOSE-29EC cells. In contrast, the expression level of bcl-2 mRNA was higher in IOSE-29EC cells than IOSE-29 cells.

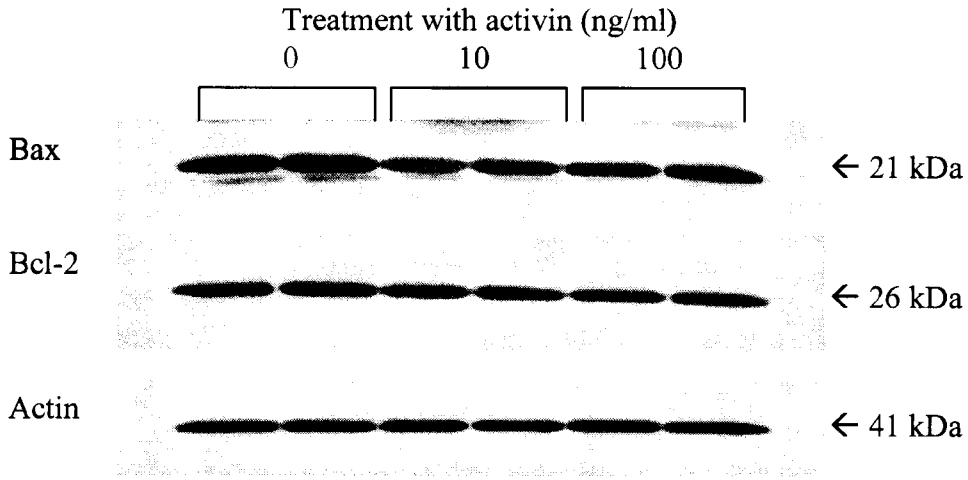


Figure 24. Effect of activin on bax and bcl-2 proteins. IOSE-29EC cells were treated with various doses of rh-activin for 24 h and immunoblot analysis was performed as described in the *Material and Methods*. Treatments with 10 and 100 ng/ml activin had no effect on both bax (21 kDa) and bcl-2 (26 kDa) proteins in these cells. The loaded amount of protein in treatment groups was normalized by actin protein (41 kDa).

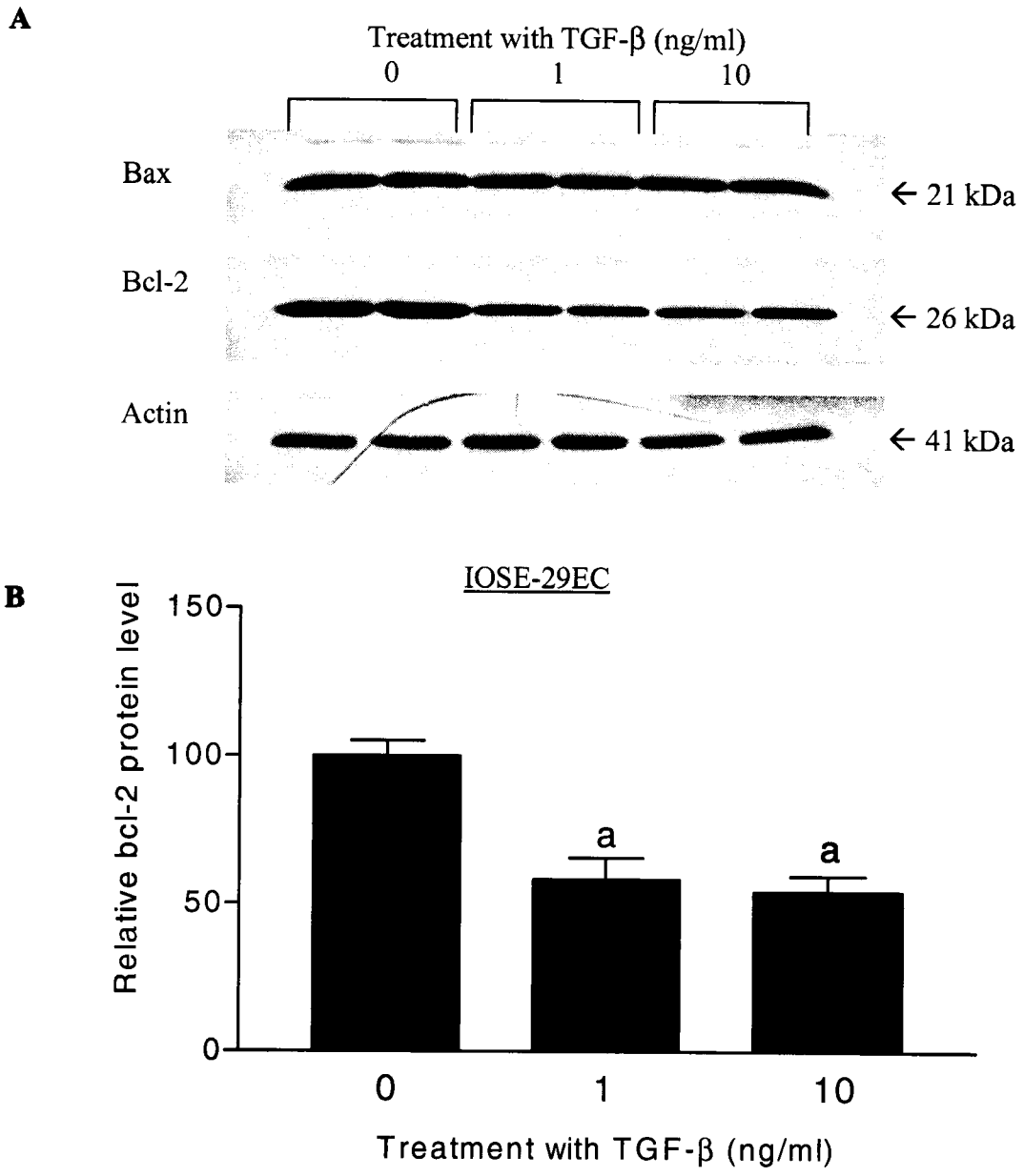


Figure 25. Effect of TGF-β on bax and bcl-2 proteins. IOSE-29EC cells were treated with various doses of TGF-β for 24 h and immunoblot analysis was performed as described in the *Material and Methods*. No significant difference of bax protein was observed in TGF-β treatment. In contrast, treatments with 1 and 10 ng/ml TGF-β induced a significant decrease of bcl-2 protein up to 50% (A and B). The loaded amount of protein in treatment groups was normalized by actin protein (41 kDa). Values are the mean ± SD for two individual experiments, each with duplicate samples. a, $P<0.05$ *vs* untreated control.

3. *EXPERIMENT C*

3.1. Expression of ERα and ERβ mRNAs

The mRNA levels of ERα and ERβ in IOSE-29 (passages 13-16), IOSE-29EC (passages 15-17), IOSE-29EC/T4 (T4) and IOSE-29EC/T5 (T5) were investigated by RT-PCR and Southern blot analysis. The possibility of cross-contamination was ruled out, because no PCR products were observed and detected in the negative control [TmA(-); without template in the RT reaction] by ethidium bromide (data not shown) and Southern blot analysis (Fig. 26). A Linear relationship between PCR products and amplification cycles was obtained in all cell types (data not shown). Predicted PCR products of GAPDH, ERα and ERβ were obtained as 373-bp, 540-bp and 279-bp respectively and confirmed by Southern blot analysis using DIG-labeled probes (Fig. 26) and sequence analysis (data not shown). This result indicates that the mRNAs of ERα and ERβ are expressed in IOSE-29, IOSE-29EC, IOSE-29EC/T4 and IOSE-29EC/T5.

3.2. Expression of ERα and ERβ proteins

To investigate the expression of ERα and ERβ proteins in immortalized OSE cell lines, immunoblot analysis was performed using the mouse monoclonal antibody for ERα and a goat polyclonal antibody for ERβ. As shown in Fig. 27 ERα protein (68 kDa) was observed in all cell types. OVCAR-3 cell line was used for positive control of the expression of ER expression. ERβ protein was also observed as 55 kDa in immortalized OSE cell lines. Immunoblot analysis of the present study demonstrated that ERα and ERβ proteins were observed in IOSE-29, IOSE-29EC, IOSE-29EC/T4 and IOSE-29EC/T5.

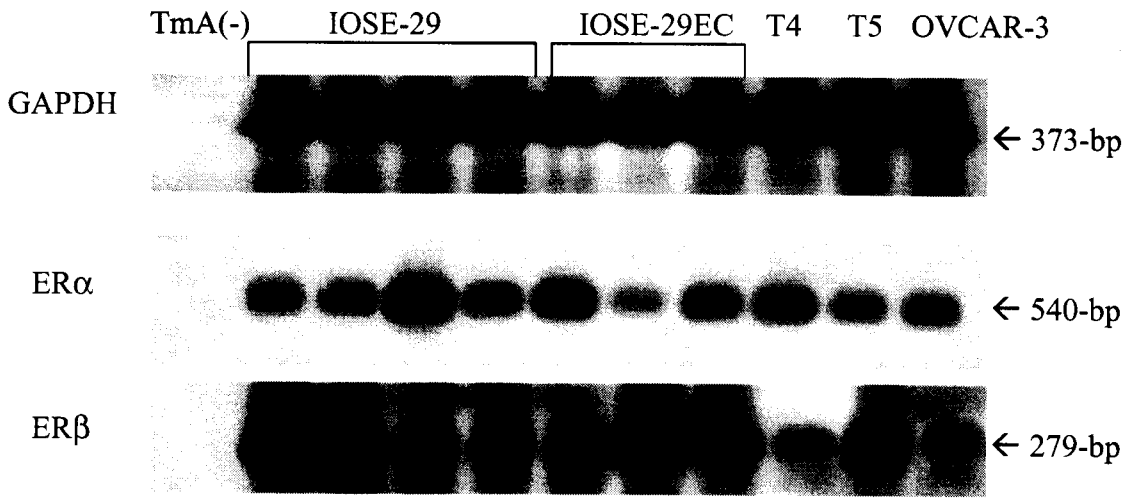


Figure 26. The mRNA levels of ERα and ERβ in IOSE cell lines. The mRNA levels of ERα and ERβ in IOSE-29 (passages 13-16), IOSE-29EC (passages 15-17), IOSE-29EC/T4 (T4) and IOSE-29EC/T5 (T5) were investigated by RT-PCR and Southern blot analysis. Expected PCR products of GAPDH, ERα and ERβ were obtained as 373-bp, 540-bp and 279-bp, respectively and confirmed by Southern blot analysis using DIG-labeled probes and sequence analysis (data not shown).

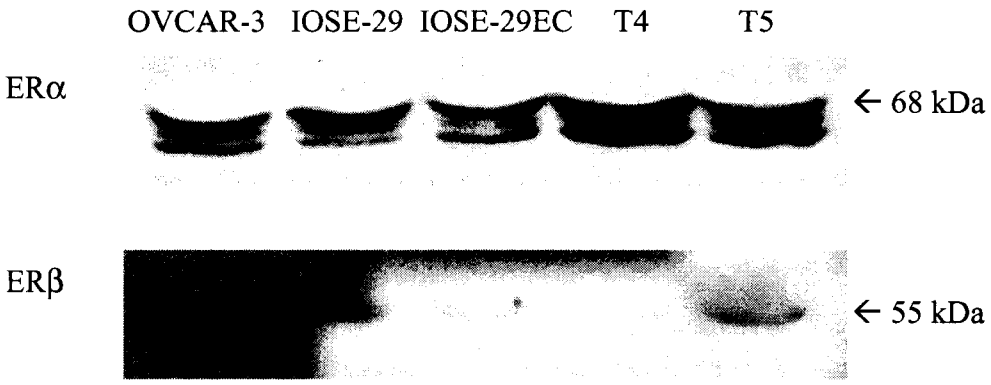
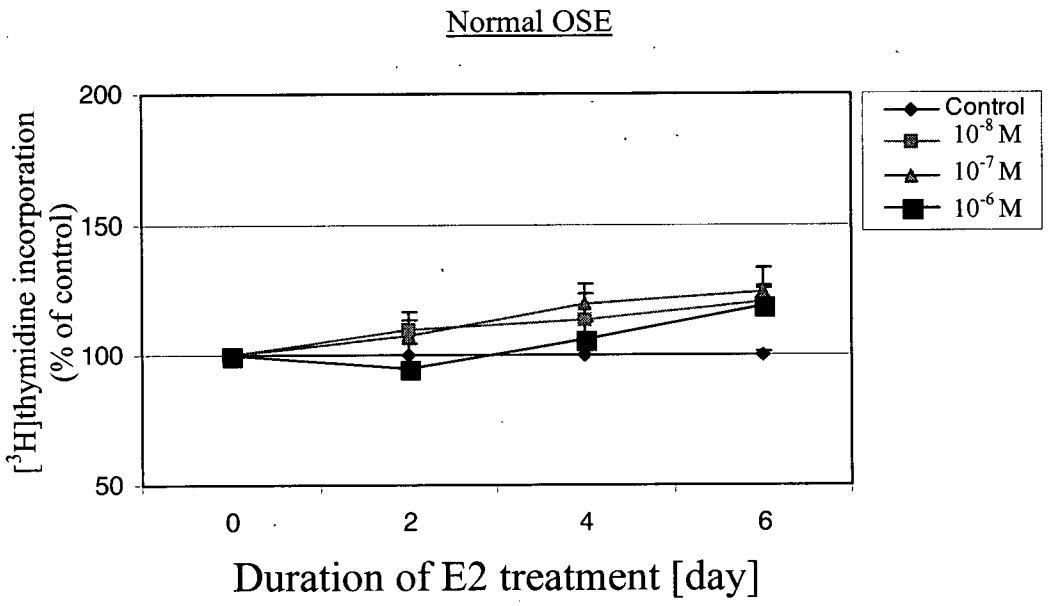


Figure 27. The protein levels of ERα and ERβ in IOSE cell lines. In parallel of mRNA expression of ERα and ERβ, immunoblot analysis was carried out using specific antibodies for ERα and ERβ in IOSE cell lines. OVCAR-3 cell line was used for positive control of the expression of ERs. ERα protein (68 kDa) was observed in all cell types. ERβ protein was also observed as 55 kDa in IOSE cell lines.

3.3. Effects of E2 on cell proliferation

To evaluate the role of E2 in normal and immortalized OSE cell lines, the cells were treated with increasing concentrations (10^{-8}, 10^{-7} and 10^{-6} M) of E2 for 2-6 days. The [^{3}H]thymidine incorporation and DNA fluorometric assays were performed as previously described in the *Materials and Methods*. Tamoxifen (Txf, 10^{-6} M), which is an estrogen antagonist, was used to block the action of E2 in the cell proliferation study. Treatment with E2 did not affect the growth of normal OSE (Fig. 28A), whereas E2 treatment for 2-6 days resulted in an increase of the growth in OVCAR-3 cells in a time-dependent manner as positive control (Fig. 28B). Highest proliferative effect of E2 was observed at 10^{-7} M, whereas the effect of E2 was less increased at 10^{-6} M in OVCAR-3 cells (Fig. 28B). No difference was observed following E2 treatment for 6 days in IOSE-29 cells by thymidine incorporation (Fig. 29A) and fluorometric assay (Fig. 29B), In contrast, treatment with E2 (10^{-8} - 10^{-6} M) prior to day 6 had no effect on thymidine incorporation, but it resulted in a significant increase on day 6 (Fig. 30A, 100.0 ± 8.16 % *vs* 134.7 ± 5.37 %, 156.8 ± 12.23 % or 132.8 ± 6.85 %) in IOSE-29EC cells. Similarly, DNA content in culture also increased significantly on day 6 (Fig. 30B, 100.0 ± 6.15 % *vs* 138.4 ± 4.08 %, 176.3 ± 22.02 % or 147.6 ± 24.00 %). Co-treatment with E2 (10^{-7} M) plus tamoxifen (10^{-6} M) attenuated the effect of E2 (100.0 ± 8.16 % *vs* 74.2 ± 6.50 % in the thymidine and 100.0 ± 6.15 % *vs* 77.7 ± 7.94 % in the DNA content) in IOSE-29EC cells. The ratio of thymidine incorporation / DNA content per culture did not change following E2 and/or tamoxifen treatments in IOSE-29 (Fig. 29, Panel C) and IOSE-29EC cells (Fig. 30, Panel C), suggesting that E2 effect does not include stimulation of proliferation. Treatment with tamoxifen only also caused inhibitory effect in IOSE-29 (Figs. 29A and B) and IOSE-29EC cells (Figs. 30A and B). In addition, a significant increase of thymidine incorporation by E2 (10^{-7} M) was also observed in IOSE-29EC/T4 and

A



B

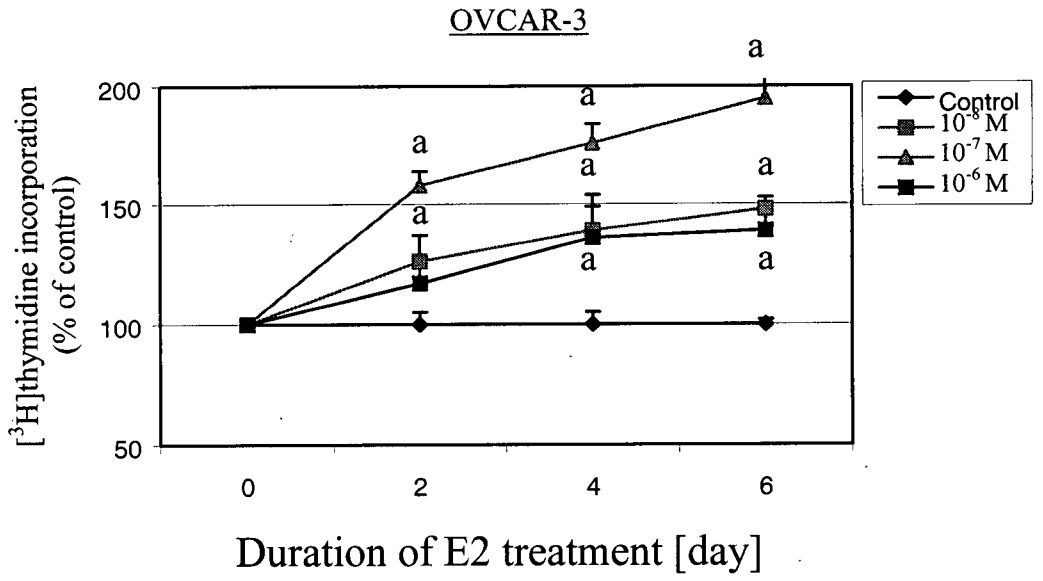
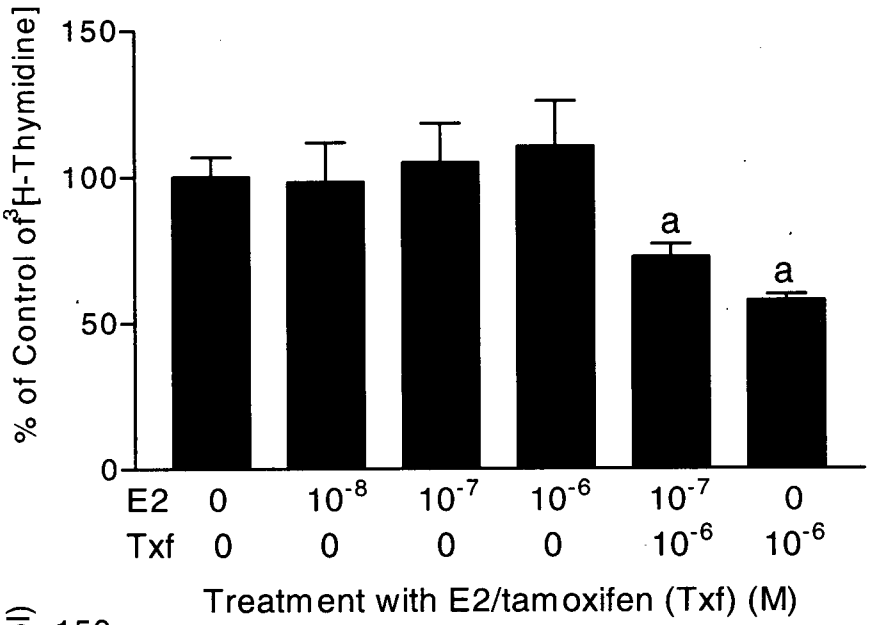


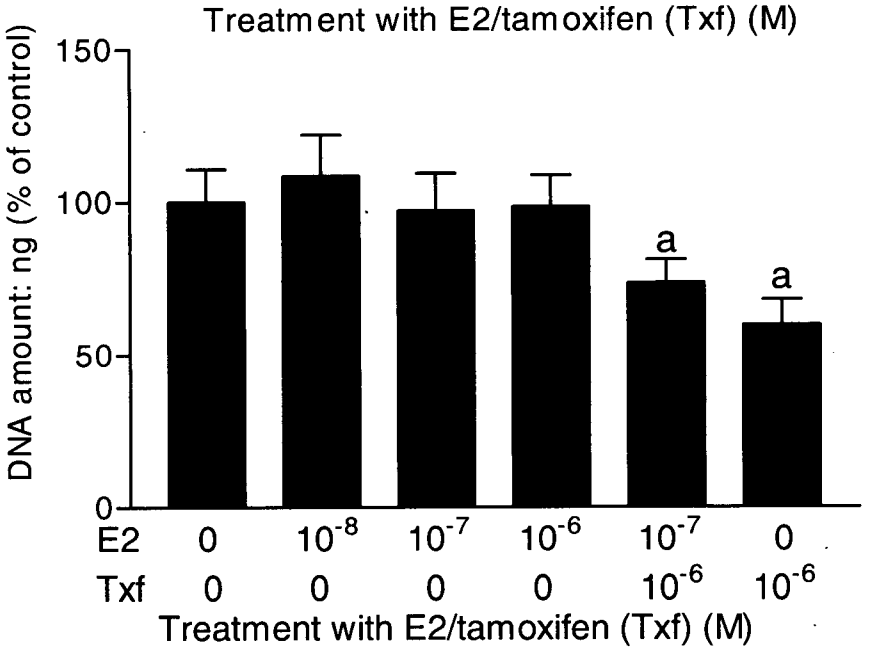
Figure 28. Effect of E2 on cell proliferation/apoptosis in normal OSE and OVCAR-3 cells. To evaluate the role of E2 in IOSE cell lines, the cells were treated with increasing concentrations (10^{-8}, 10^{-7} and 10^{-6} M) of E2 and/or tamoxifen (10^{-6} M) for 2-6 days as previously described in the *Materials and Methods*. [^{3}H]thymidine incorporation was analyzed following E2 treatment for 2-6 days in normal OSE (A) and OVCAR-3 cells (B).

A



IOSE-29

B



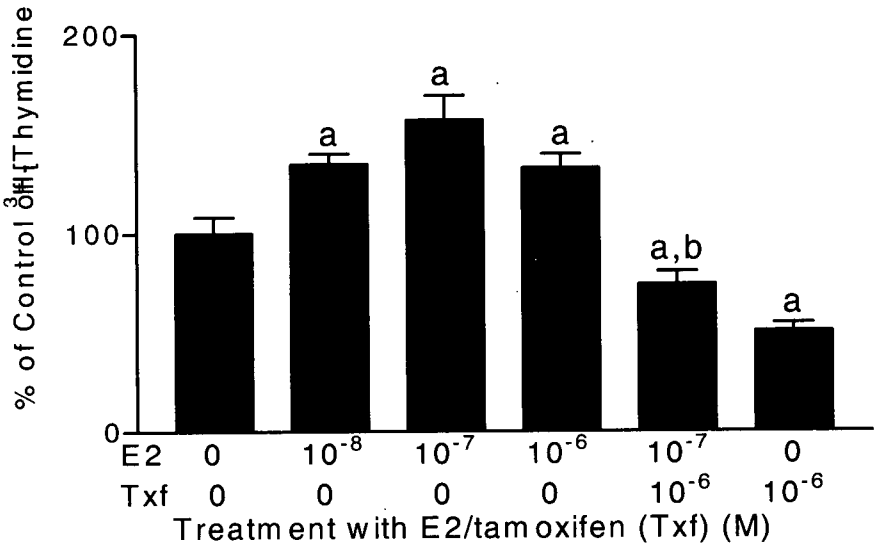
C

The ratio of thymidine/DNA content

E2 (M)	0	10^{-8}	10^{-7}	10^{-6}	10^{-7}	0
Txf (M)	0	0	0	0	10^{-6}	10^{-6}
	1.00	0.90	1.08	1.12	0.99	0.96

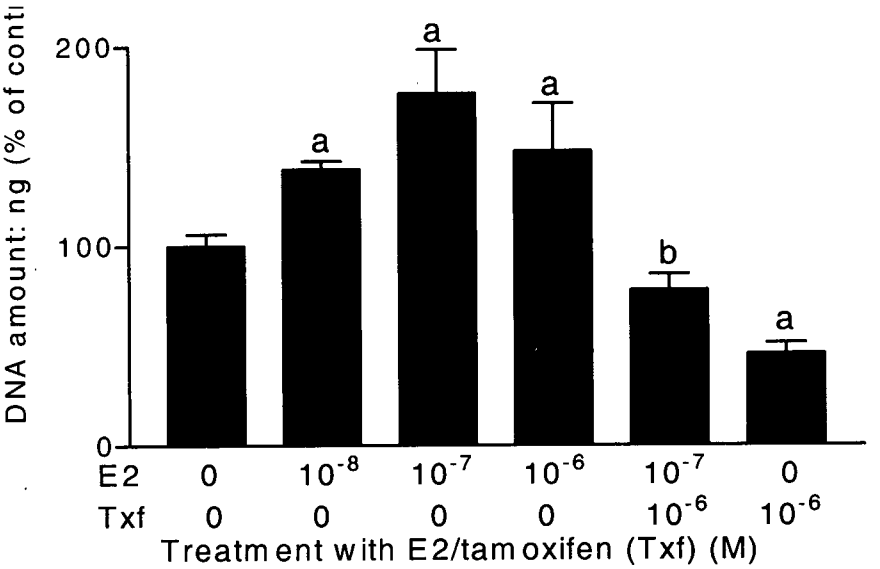
Figure 29. Effect of E2 on cell proliferation/apoptosis in IOSE-29 cells. A [^{3}H]thymidine incorporation (A) and DNA fluorometic assays (B) were performed following E2/tamoxifen treatment for 6 days in IOSE-29. The ratio of thymidine incorporation / DNA content per culture did not change following E2 and/or tamoxifen treatments in IOSE-29 (Panel C). Data are shown as the means of three individual experiments with triplicate, and are presented as the mean ± SD. a, $P<0.05$ *vs.* untreated control.

A



IOSE-29EC

B



C

The ratio of thymidine / DNA content

E2 (M)	0	10^{-8}	10^{-7}	10^{-6}	10^{-7}	0
Txf (M)	0	0	0	0	10^{-6}	10^{-6}
	1.00	0.97	0.89	0.90	0.96	1.11

Figure 30. Effect of E2 on cell proliferation/apoptosis in IOSE-29EC cells. A [^{3}H]thymidine incorporation (A) and DNA fluorometic assays (B) were performed following E2/tamoxifen treatment for 6 days in IOSE-29EC. The ratio of thymidine incorporation / DNA content per culture did not change following E2 and/or tamoxifen treatments in IOSE-29EC (Panel C). Data are shown as the means of three individual experiments with triplicate, and are presented as the mean ± SD. a, $P<0.05$ *vs.* untreated control; b, $P<0.05$ *vs.* E2 (10^{-7} M) treatment.

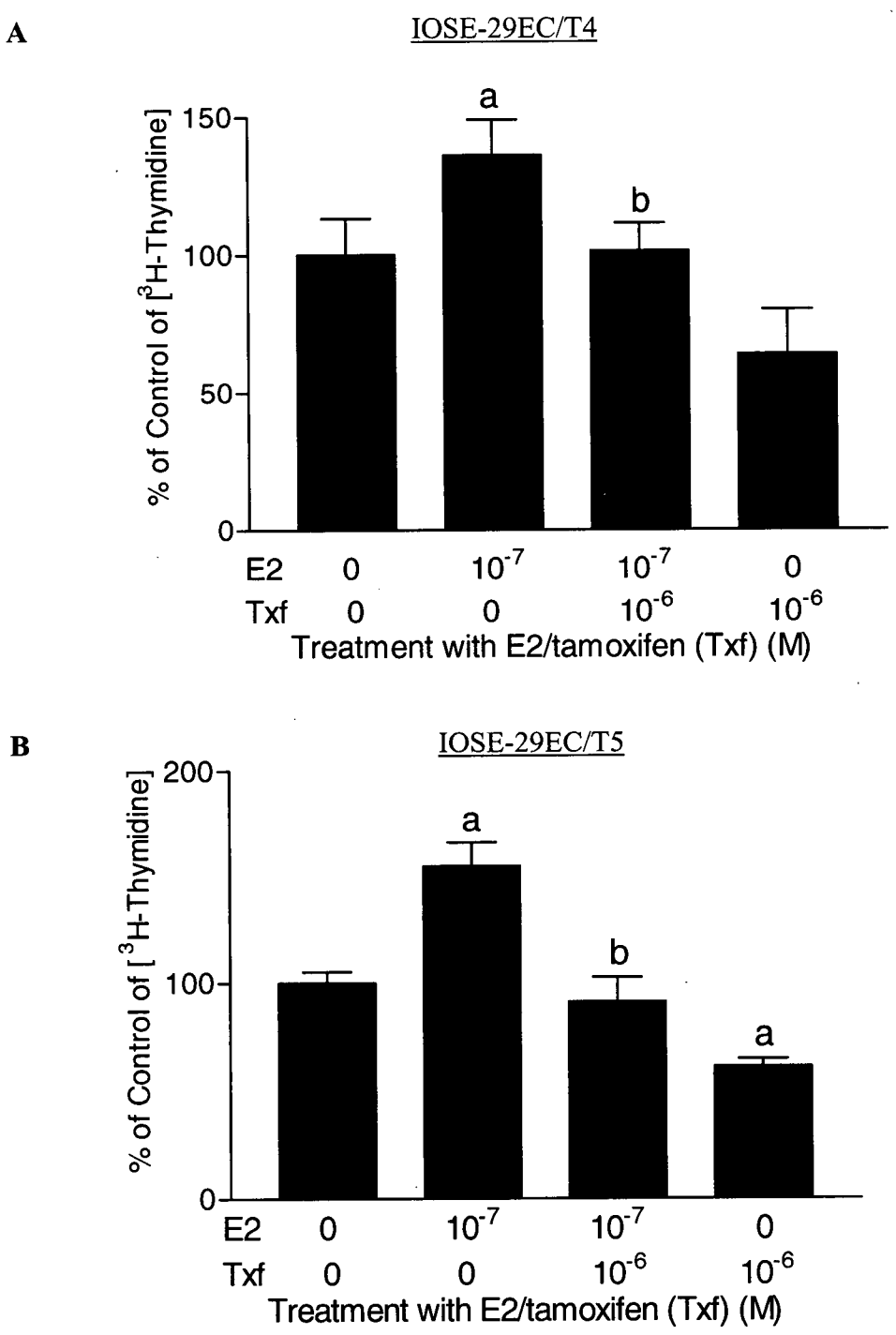


Figure 31. Effect of E2 on cell proliferation/apoptosis in IOSE-29EC/T4 and /T5. A thymidine incorporation by E2/tamoxifen was also analyzed in IOSE-29EC/T4 (A) and IOSE-29EC/T5 (B) cells after 6-day treatment. Data are shown as the means of three individual experiments with triplicate, and are presented as the mean ± SD. a, $P<0.05$ *vs.* untreated control; b, $P<0.05$ *vs.* E2 (10^{-7} M) treatment.

IOSE-29EC/T5 cells, whereas co-treatment with tamoxifen attenuated the E2 effect (Figs. 31A and B).

3.4. Effects of E2 on apoptosis

To examine the role of E2 in the prevention of apoptosis, DNA fragmentation was measured using the cell death detection ELISA. To quantify the induction of apoptosis, IOSE-29EC cells were treated with tamoxifen (10^{-6} M) and/or E2 (10^{-8}, 10^{-7} and 10^{-6} M) for 6 days. As shown in Fig 32, treatment with tamoxifen resulted in a significant increase of DNA fragmentation in IOSE-29EC cells (100.0 ± 5.89 % *vs* 287.7 ± 11.26 %). Co-treatments with E2 (10^{-8}, 10^{-7} and 10^{-6} M) plus tamoxifen attenuated tamoxifen-induced apoptosis in a dose-dependent manner (Fig. 32, 287.7 ± 11.26 % *vs* 219.8 ± 21.47 %, 175.8 ± 12.02 % or 174.9 ± 16.50 %).

3.5. Expression of pro- and anti-apoptotic gene mRNAs and proteins

The mRNA levels of pro-apoptotic bax and anti-apoptotic bcl-2 in IOSE-29 and IOSE-29EC were investigated by RT-PCR and Southern blot analysis. Predicted PCR products of GAPDH, bax and bcl-2 were obtained as 373-bp, 323-bp and 459-bp respectively and confirmed by Southern blot analysis using DIG-labeled probes (Fig. 33) and sequence analysis (data not shown). No difference was observed in the expression level of bax mRNA between IOSE-29 and IOSE-29EC cells. In contrast, the mRNA expression level of bcl-2 was higher in IOSE-29EC cells than IOSE-29 cells (Fig. 33A). In parallel of mRNA level, protein level of bcl-2 was investigated in these cell lines. As shown in Fig. 5B, the expression level of bcl-2 protein was higher in IOSE-29EC cells than IOSE-29 cells.

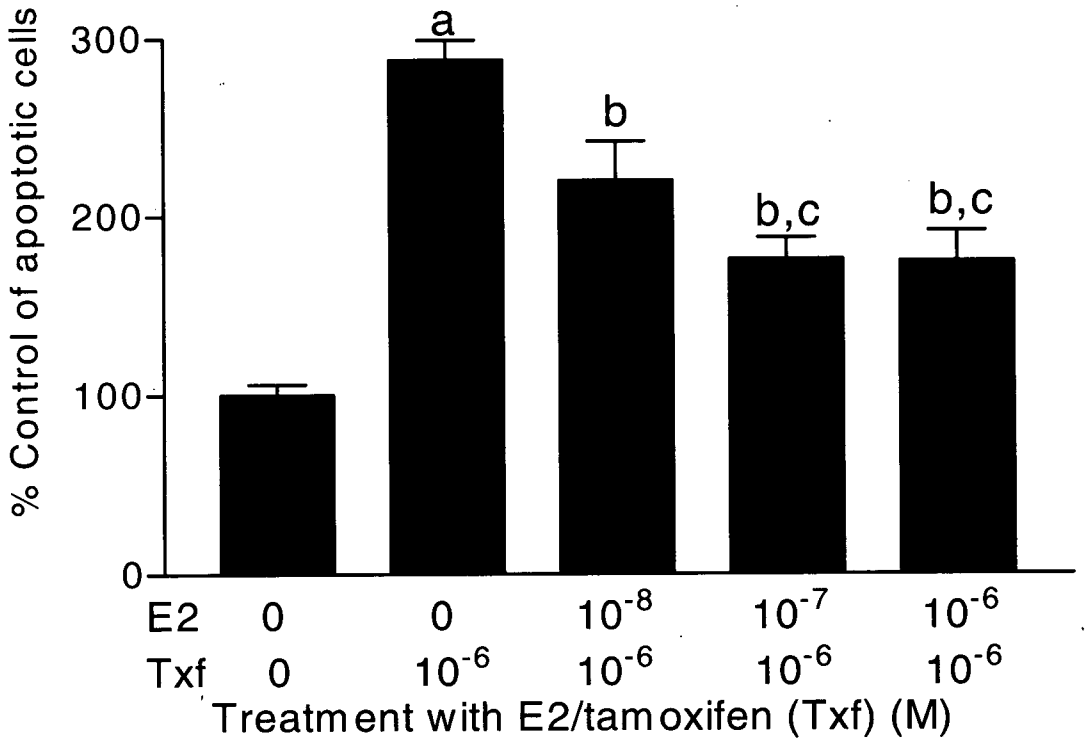


Figure 32. Effect of E2 on apoptosis in IOSE-29EC cells. To examine the role of E2 in the prevention of apoptosis, DNA fragmentation was measured using the cell death detection ELISA. Data are shown as the means of three individual experiments with duplicate, and are presented as the mean ± SD. a, $P<0.05$ *vs.* untreated control; b, $P<0.05$ *vs.* tamoxifen (10^{-6} M) treatment; c, $P<0.05$ *vs.* E2 (10^{-8} M) plus tamoxifen (10^{-6} M) treatment.

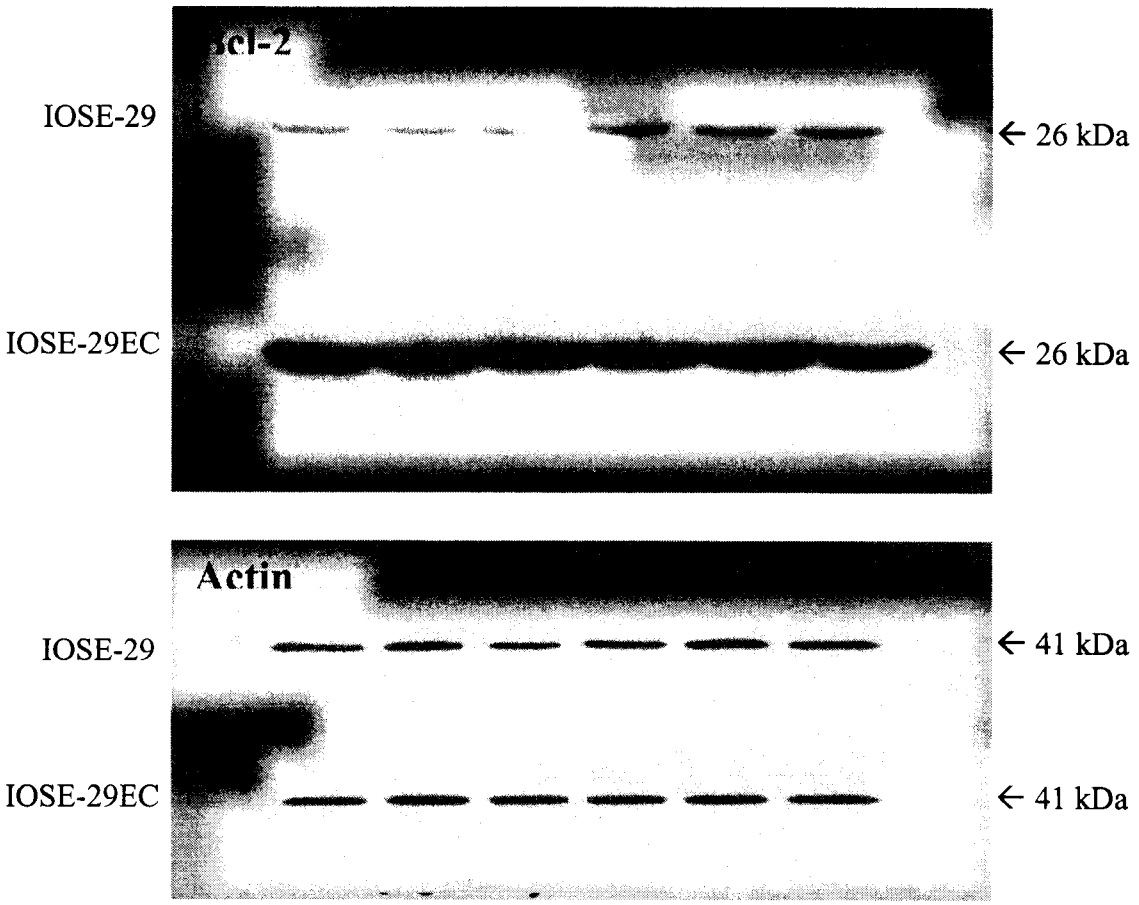


Figure 33. Expression of bcl-2 protein in IOSE-29 and IOSE-29EC cells. The protein levels of bax and bcl-2 in IOSE-29 and IOSE-29EC were investigated by immunoblot analysis. The protein amount was normalized by actin protein (41 kDa).

3.6. Effect of E2 on pro- and anti-apoptotic mRNAs

To investigate the mechanism of E2 action in the prevention of apoptosis, the regulation of bax and bcl-2 was examined using RT-PCR in IOSE-29EC cells. The cells were treated with E2 and/or tamoxifen for 24 h. The expected sizes of PCR products for bax and bcl-2 were obtained as 323-bp and 459-bp respectively (Fig. 34). The mRNA expression of bax and bcl-2 was normalized with GAPDH (373-bp) to quantify the mRNA levels. Treatments with E2 (10^{-8} to 10^{-6} M) up-regulated bcl-2 mRNA up to 2-fold in these cells (Figs. 34 and 35, 100.0± 7.19 % *vs* 172.9 ± 14.47 %, 190.9 ± 22.03 % or 171.8 ± 17.55 %). Co-treatment with tamoxifen (10^{-6} M) plus E2 attenuated this E2 effect (190.9 ± 22.03 % *vs* 116.8 ± 10.92 %) as shown in Figs. 34 and 35. However, no significant difference in the mRNA level of bax was observed by E2 treatment (Fig. 34).

3.7. Effect of E2 on pro- and anti-apoptotic proteins

To investigate the protein levels of bax and bcl-2 by E2, immunoblot analysis was performed following estrogen and/or tamoxifen treatments. The cells were treated with E2 and/or tamoxifen for 48 h as previously described in the *Material and Methods*. Specific signals for bax and bcl-2 protein were detected at 21 kDa and 26 kDa, respectively, as shown in Fig. 36. Consistent with the mRNA levels, treatments with E2 (10^{-8} to 10^{-6} M) significantly up-regulated bcl-2 protein level in these cells (Figs. 36 and 37, 100.0 ± 9.92 % *vs* 162.05 ± 12.68 %, 166.7 ± 19.61 % or 154.4 ± 20.86 %). Co-treatment with tamoxifen (10^{-6} M) plus E2 attenuated this E2 effect (166.7 ± 19.61 % *vs* 109.9 ± 10.22 %) as shown in Fig. 37. In contrast, no significant difference in the protein level of bax was observed by E2 treatment in IOSE-29EC cells (Fig. 36).

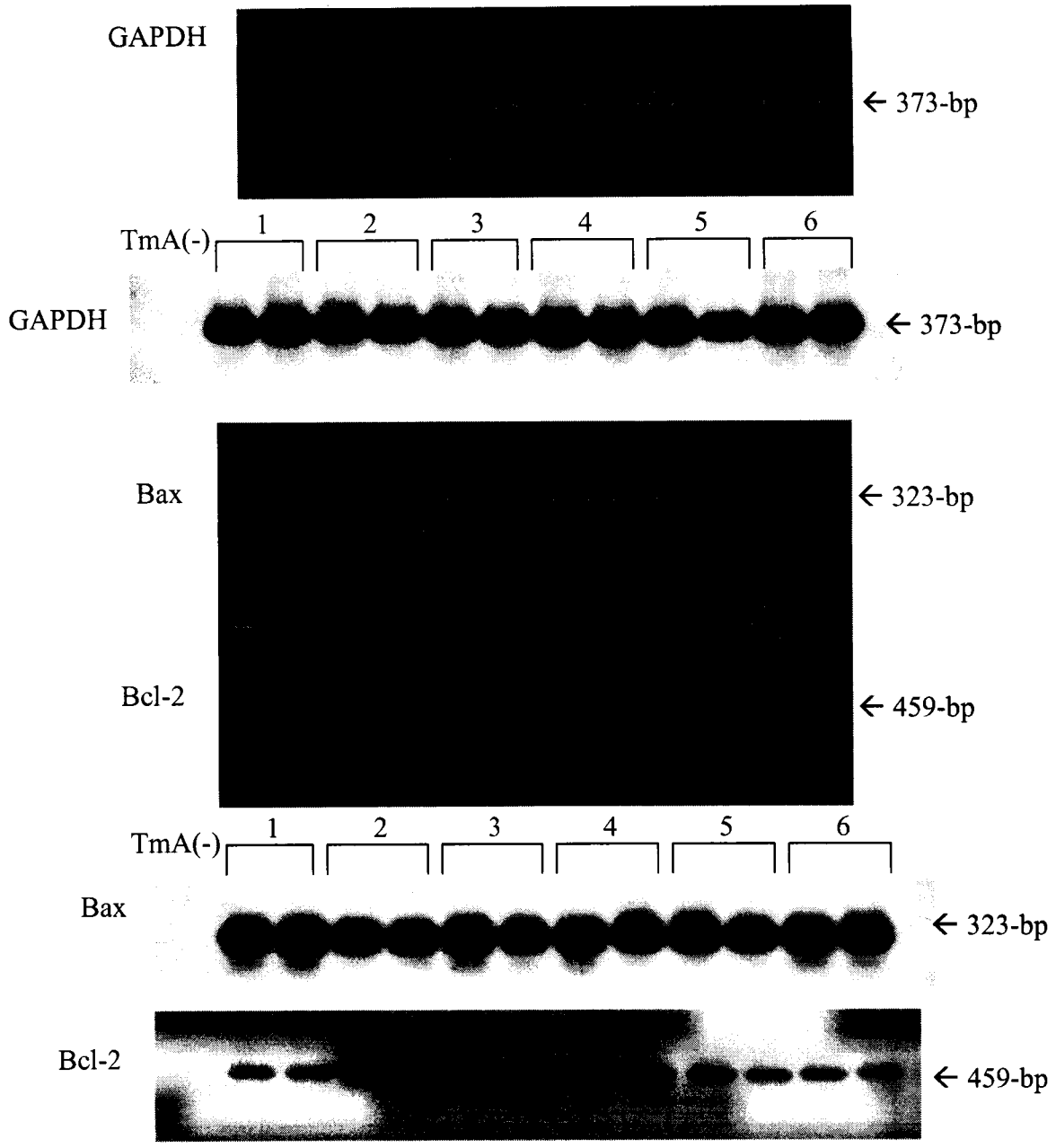


Figure 34. Effect of E2 on bax and bcl-2 mRNA levels in IOSE-29EC cells. To investigate the mechanism of E2 in the prevention of apoptosis, the regulation of bax and bcl-2 was examined using RT-PCR in IOSE-29EC cells. The cells were treated with E2 and/or tamoxifen (Txf) for 24 h. The expected sizes of PCR products for bax and bcl-2 were obtained as 323-bp and 459-bp respectively. The mRNA expression levels of bax and bcl-2 were normalized with GAPDH (393-bp) to quantify the mRNA levels. 1, untreated control; 2, E2 (10^{-8} M) treatment; 3, E2 (10^{-7} M) treatment; 4, E2 (10^{-6} M) treatment; 5, tamoxifen (10^{-6} M) treatment; 6, E2 (10^{-7} M) plus tamoxifen (10^{-6} M) treatment.

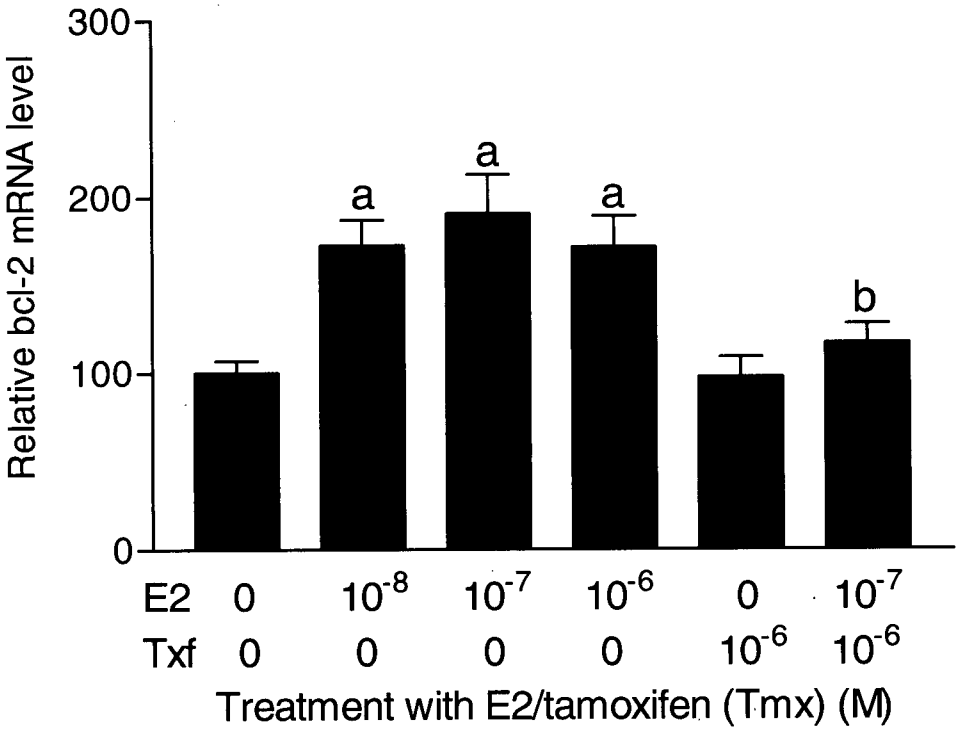


Figure 35. Effect of E2 on bcl-2 mRNA level in IOSE-29EC cells. A relative bcl-2 mRNA expression level was examined after treatment E2 and/or tamoxifen. Data are shown as the means of three individual experiments, and are presented as the mean ± SD. a, $P<0.05$ *vs.* untreated control; b, $P<0.05$ *vs.* E2 (10^{-7} M) treatment

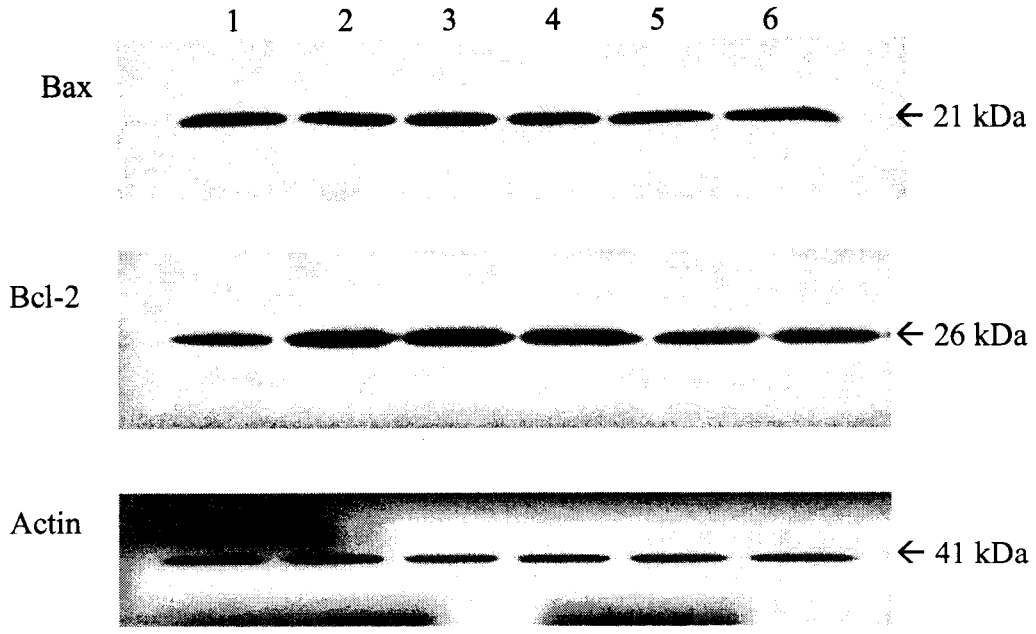


Figure 36. Effect of E2 on bax and bcl-2 proteins in IOSE-29EC cells. In parallel of mRNA level, protein level of bax and bcl-2 was investigated by immunoblot analysis. The cells were treated with E2 and/or tamoxifen (Txf) for 48 h as previously described in the *Materials and Methods*. Specific signals for bax and bcl-2 protein were detected at 21 kDa and 26 kDa respectively. The protein amount in the groups was normalized by actin protein (41 kDa). 1, untreated control; 2, E2 (10^{-8} M) treatment; 3, E2 (10^{-7} M) treatment; 4, E2 (10^{-6} M) treatment; 5, tamoxifen (10^{-6} M) treatment; 6, E2 (10^{-7} M) plus tamoxifen (10^{-6} M) treatment.

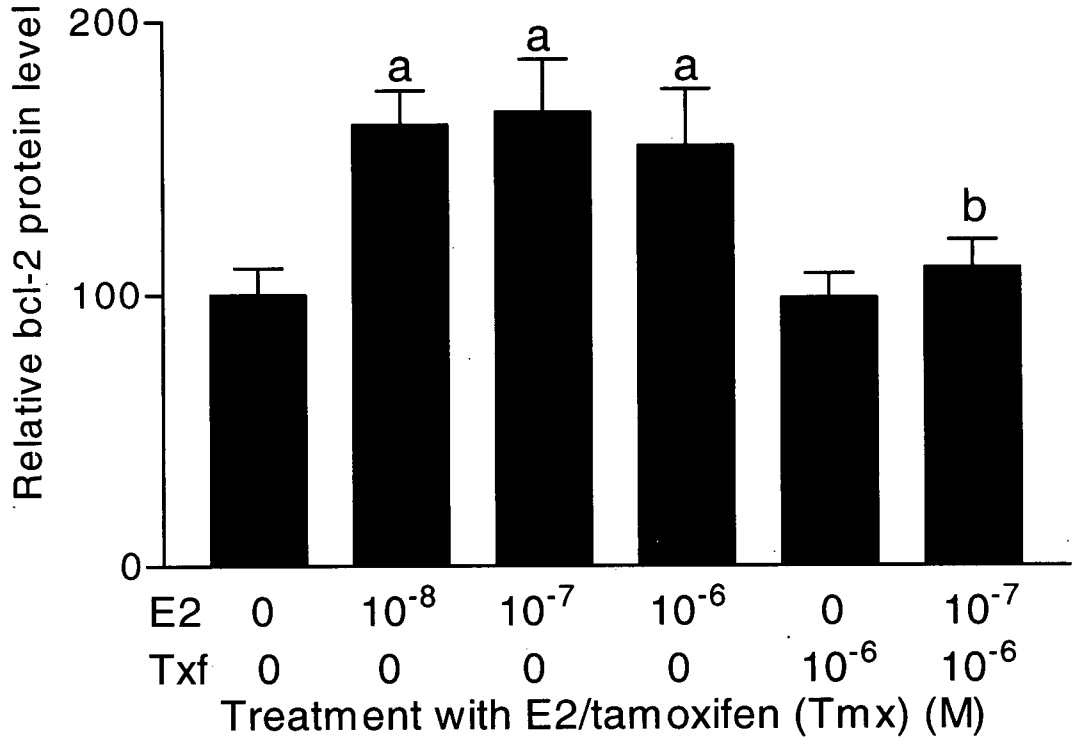


Figure 37. Effect of E2 on bcl-2 protein in IOSE-29EC cells. In parallel of mRNA level, protein level of bax and bcl-2 was investigated by immunoblot analysis. The cells were treated with E2 and/or tamoxifen (Txf) for 48 h as previously described in the *Materials and Methods*. The protein amount in the groups was normalized by actin protein (41 kDa). Data are shown as the means of three individual experiments, and are presented as the mean ± SD. a, $P<0.05$ *vs.* untreated control; b, $P<0.05$ *vs.* E2 (10^{-7} M) treatment.

4. *EXPERIMENT D*

4.1. Expression of FSH-R mRNA

The mRNA expression of FSH-R in normal and neoplastic OSE cells was investigated by RT-PCR, Southern blot and Northern blot analysis. A predicted PCR product of FSH-R was obtained as 369-bp and confirmed by Southern blot analysis using DIG-labeled probes (Fig. 38) and sequence analysis (data not shown). The human granulosa luteal cells (hGLCs) were used for positive control. As demonstrated in Fig. 38, the FSH-R mRNAs are expressed in normal OSE and IOSE cell lines (IOSE-29, IOSE-29EC, IOSE-29EC/T4 and IOSE-29EC/T5).

To confirm FSH-R mRNA expression by RT-PCR and Southern blot, Northern blot analysis was performed in IOSE-29, IOSE-29EC and ovarian cancer cell lines, including OVCAR-3 and SKOV-3 cells (Fig. 39). As seen in Fig. 39, the low levels of two transcripts (4.1 and 2.4-kb) of FSH-R mRNA were demonstrated in IOSE cell lines (IOSE-29 and IOSE-29EC) and two ovarian cancer cell lines (OVCAR-3 and SKOV-3). The predominant transcript was 4.1-kb in size as shown in Fig. 39 (Minegishi *et al.*, 1997). The high level of FSH-R mRNA transcripts was observed in human granulosa-luteal cells as positive control.

4.2. Effects of FSH on proliferative index

To evaluate the role of FSH in normal and immortalized OSE cell lines, the cells were treated with increasing concentrations (10, 100 and 1000 ng/ml) of human recombinant FSH for 24 h and a [^{3}H]thymidine incorporation assay was performed as previously described (30). Treatments with increasing doses of FSH (10, 100 or 1000 ng/ml) resulted in a significant growth-stimulation in normal OSE (Fig. 40A, 100.0 ± 8.33 % *vs.* 135.1 ± 7.49, 137.1 ± 9.06 or 135.4 ± 13.90) and OVCAR-3 cells (Fig. 40B, 100.0 ± 9.38 % *vs.* 128.4 ± 7.21 or 128.9 ± 9.60).

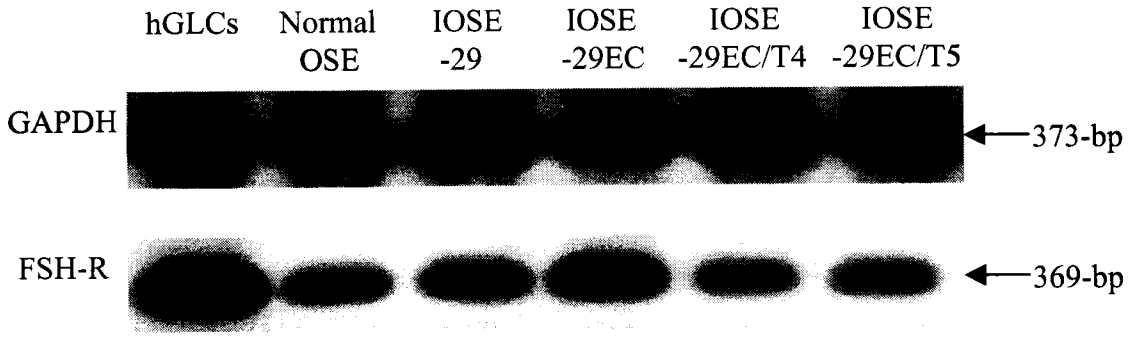


Figure 38. Expression of FSH-R mRNA in normal OSE and IOSE cell lines. The mRNA expression of FSH-R in normal and neoplastic OSE cells was investigated by RT-PCR and Southern blot analysis. A predicted PCR product of FSH-R was obtained as 369-bp and confirmed by Southern blot analysis using DIG-labeled probes and sequence analysis (data not shown). The amplification of GAPDH (373-bp) was performed to rule out the possibility of RNA degradation, and was used to control the variation in mRNA concentration in the PCR reaction.

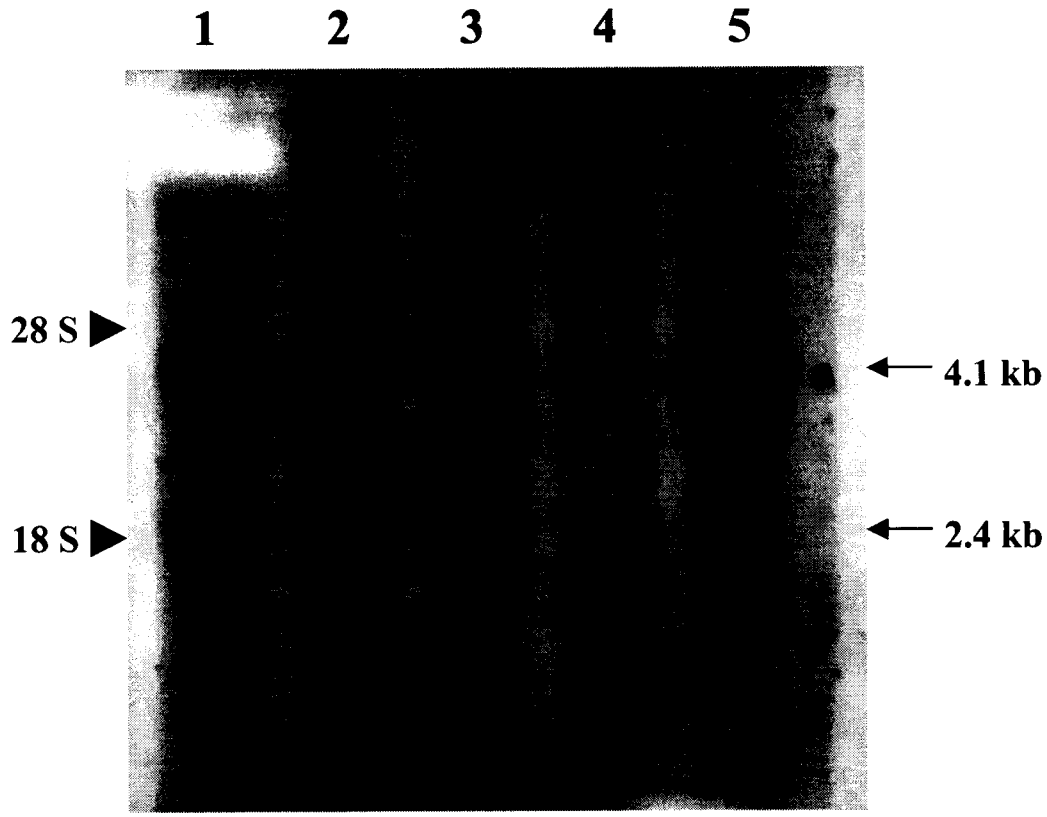
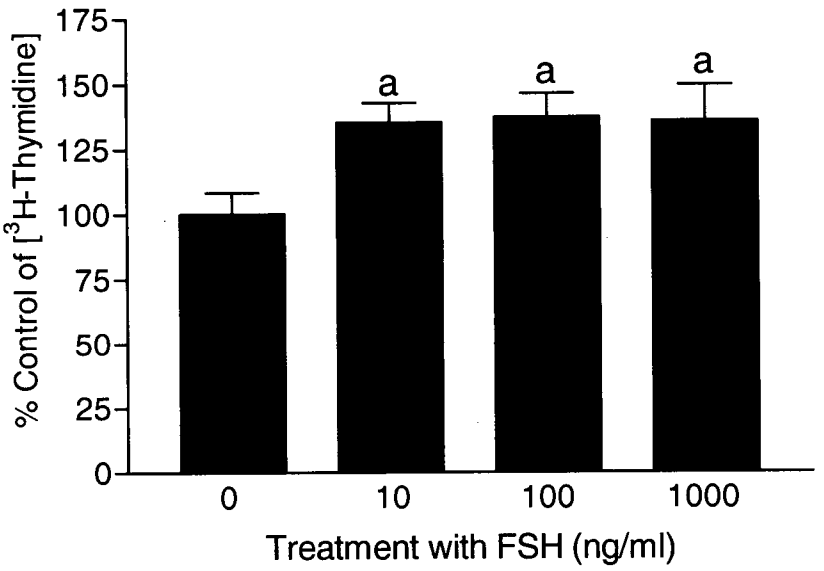


Figure 39. Expression of FSH-R mRNA in IOSE and ovarian cancer cell lines. The mRNA expression of FSH-R in neoplastic OSE cells was investigated by Northern blot analysis. Total RNA (50 μg) was prepared and resolved by formaldehyde denaturing agarose gel electrophoresis. The hybridization was performed using a radioactive labeled FSH-R probe. 1, IOSE-29; 2, IOSE-29EC; 3, OVCAR-3; 4, SKOV-3; 5, Human granulosa-luteal cells (positive control).

A



B

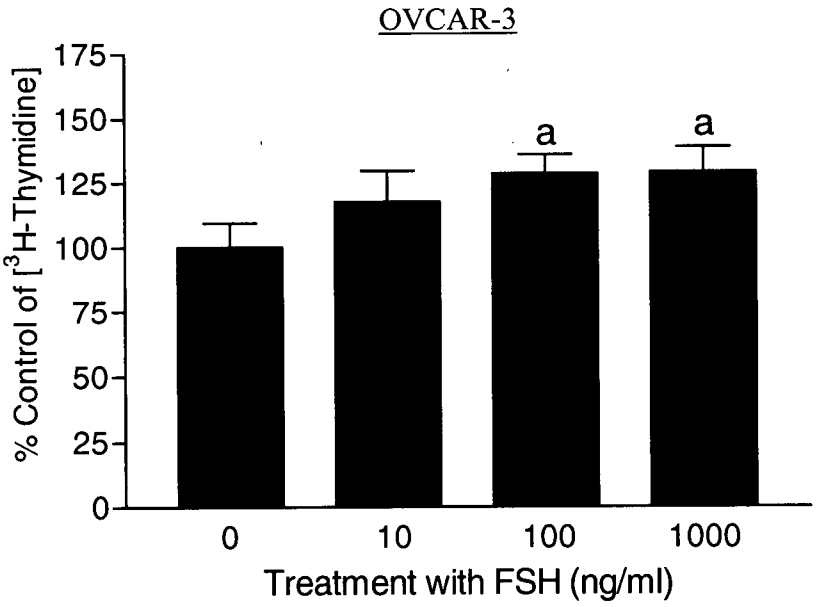


Figure 40. Effects of FSH on cell proliferation in normal OSE and OVCAR-3 cells. The cells were treated with increasing concentrations (10, 100 and 1000 ng/ml) of human recombinant FSH for 24 h and a [^{3}H]thymidine incorporation assay was performed as previously described in the *Materials and Methods*. Treatments with increasing doses of FSH (10, 100 or 1000 ng/ml) resulted in a significant growth-stimulation in normal OSE (A) and OVCAR-3 cells (B). Data are shown as the means of three individual experiments with triplicate, and are presented as the mean ± SD. a, $P<0.05$ *vs.* untreated control.

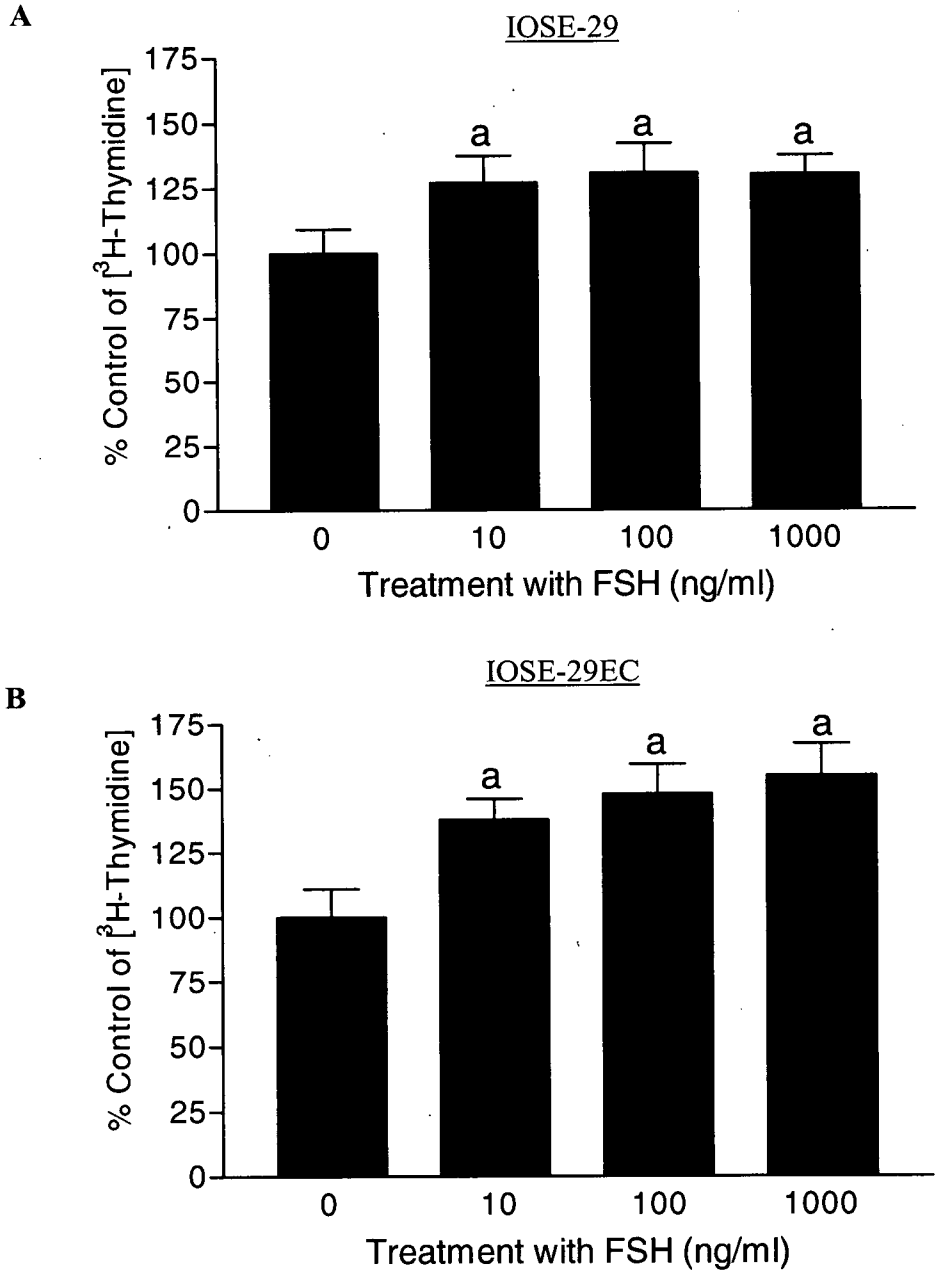


Figure 41. Effects of FSH on cell proliferation in IOSE-29 and IOSE-29EC cells. The cells were treated with increasing concentrations (10, 100 and 1000 ng/ml) of human recombinant FSH for 24 h and a [^{3}H]thymidine incorporation assay was performed as previously described in the *Materials and Methods*. Treatments with increasing doses of FSH (10, 100 or 1000 ng/ml) resulted in a significant growth-stimulation in IOSE-29 (A) and IOSE-29EC cells (B). Data are shown as the means of three individual experiments with triplicate, and are presented as the mean ± SD. a, $P<0.05$ *vs.* untreated control.

In addition, treatments with same concentrations of FSH induced a significant growth-stimulation of IOSE-29EC (Fig. 41A, 100.0 ± 9.08 % *vs.* 126.9 ± 10.12, 130.6 ± 11.23 or 129.9 ± 7.25) and IOSE-29EC cells (Fig. 41B, 100.0 ± 11.05 % *vs.*137.5 ± 8.21, 147.6 ± 11.65 or 154.6 ± 12.45). This result indicates that both normal and neoplastic OSE cells are responsive to FSH treatments, which resulted in a growth-stimulation in these cells.

4.3. Expression of MAPKs in normal and neoplastic OSE cells

The basal expression level of phosphorylated p44/p42 MAPK (P-MAPK) normalized by total MAPK (T-MAPK) was compared using the same amount of protein (30 μg) in normal OSE, IOSE cell lines (IOSE-29 and IOSE-29EC) and ovarian cancer cell lines (CaOV-3, OVCAR-3 and SKOV-3). As shown in Fig. 42, normal OSE cells expressed a high level of P-MAPK. Interestingly, the basal level of P-MAPK in CaOV-3 cells was very low, when compared with other cell lines (Fig. 42).

4.4. Effects of FSH and/or PD98059 on MAPK activation

To investigate the role of FSH on MAPK activation, the cells were pretreated with 50 μM PD98059, a MAPK/ERK kinase (MEK) inhibitor, for 30 min, followed by treatment with increasing doses of FSH (10, 100 or 1000 ng/ml) for 10 min. As shown in Figs. 43A and B, treatments with FSH induced a significant increase in MAPK activation in IOSE-29 cells (100.0 ± 9.94 % *vs.*147.8 ± 9.31, 151.9 ± 10.62 or 156.7 ± 9.83). The stimulatory effect of FSH was completely reversed by pretreatment with PD98059 (157.3 ± 11.21 % *vs.*56.3 ± 5.18). Similarly, treatments with FSH resulted in a significant increase in MAPK activation in IOSE-29EC cells (Figs. 44A and B, 100.0 ± 7.54 % *vs.*182.9 ± 11.84, 183.4 ± 9.52 or 179.2 ± 9.00). This stimulatory effect of FSH was completely blocked by pretreatment with PD98059 (179.3 ± 12.04 % *vs.* 65.7 ± 4.98). Treatment with PD98059 alone resulted in a significant decrease of basal P-MAPK in both IOSE-29 and IOSE-29EC cells (Figs. 43B and 44B).

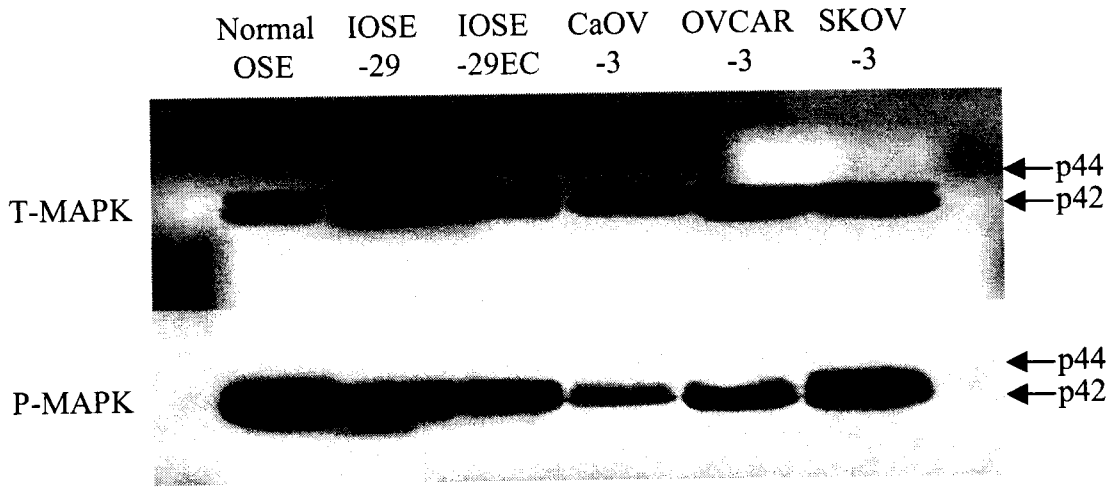


Figure 42. Basal expression level of P-MAPK and T-MAPK in normal and neoplastic OSE cells. The P-MAPK normalized by T-MAPK was compared after running the same amount of protein (30 μg) in normal OSE, IOSE cell lines (IOSE-29 and IOSE-29EC) and ovarian cancer cell lines (CaOV-3, OVCAR-3 and SKOV-3).

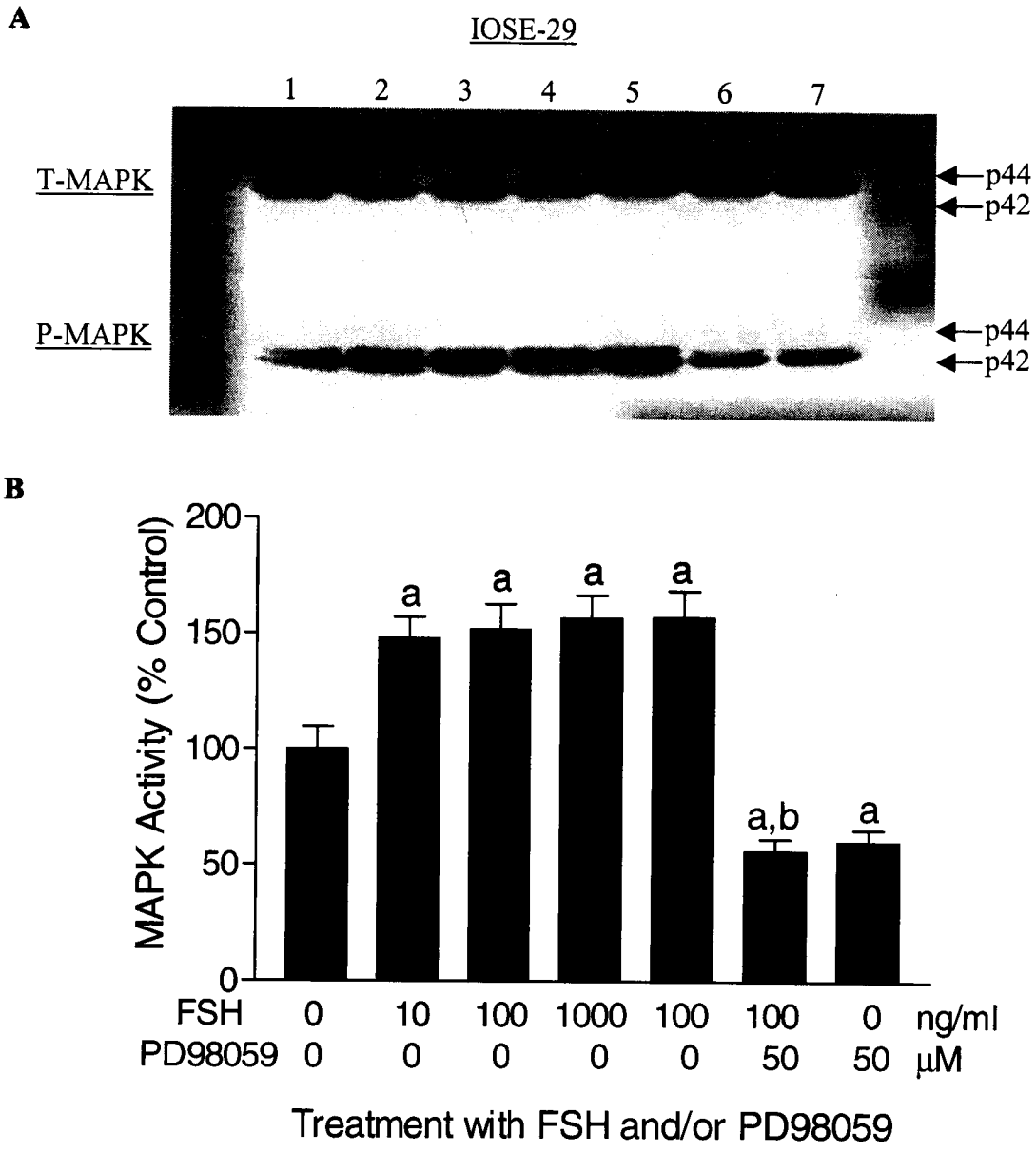


Figure 43. Effects of FSH in the presence or absence of PD98059 on MAPK activation IOSE-29 cells. To investigate the role of FSH on MAPK, the cells were pretreated with 50 μM PD98059, a MAPK/ERK kinase (MEK) inhibitor, for 30 min, followed by treatment with increasing doses of FSH (10, 100 or 1000 ng/ml) for 10 min. The P-MAPK normalized by T-MAPK was analyzed in IOSE-29 (A and B). Data are shown as the means of three individual experiments, and are presented as the mean ± SD. a, $P<0.05$ *vs.* untreated control; b, $P<0.05$ *vs.* FSH (100 ng/ml) treatment; c, $P<0.05$ *vs.* PD98059 (50 μM) treatment. 1, untreated control; 2, FSH (10 ng/ml) treatment; 3, FSH (100 ng/ml) treatment; 4, FSH (1000 ng/ml) treatment; 5, FSH (100 ng/ml) treatment; 6, FSH (100 ng/ml) plus PD98059 (50 μM) treatment; 7, PD98059 (50 μM) treatment.

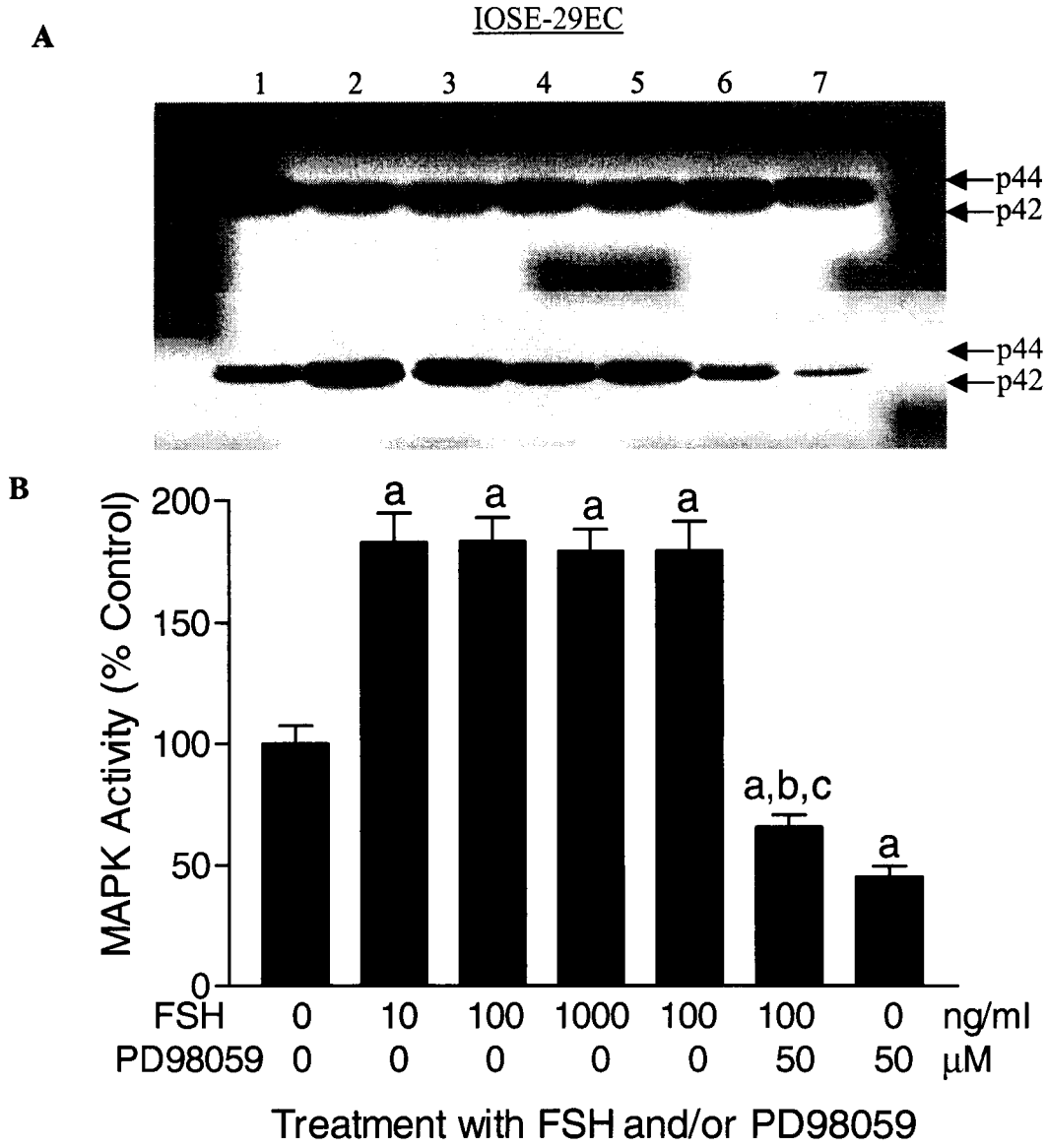


Figure 44. Effects of FSH in the presence or absence of PD98059 on MAPK activation IOSE-29EC cells. To investigate the role of FSH on MAPK, the cells were pretreated with 50 μM PD98059, a MAPK/ERK kinase (MEK) inhibitor, for 30 min, followed by treatment with increasing doses of FSH (10, 100 or 1000 ng/ml) for 10 min. The P-MAPK normalized by T-MAPK was analyzed in IOSE-29EC (A and B). Data are shown as the means of three individual experiments, and are presented as the mean ± SD. a, $P<0.05$ *vs.* untreated control; b, $P<0.05$ *vs.* FSH (100 ng/ml) treatment; c, $P<0.05$ *vs.* PD98059 (50 μM) treatment. 1, untreated control; 2, FSH (10 ng/ml) treatment; 3, FSH (100 ng/ml) treatment; 4, FSH (1000 ng/ml) treatment; 5, FSH (100 ng/ml) treatment; 6, FSH (100 ng/ml) plus PD98059 (50 μM) treatment; 7, PD98059 (50 μM) treatment.

Time-dependent experiment was performed following treatment with FSH (100 ng/ml) and/or pretreatment with PD98059 (50 μM) on MAPK activity. As shown in Figs. 45A and B, treatment with FSH induced a significant increase in P-MAPK at 5-10 min in IOSE-29 cells (100.0 ± 9.15 % *vs.* 132.6 ± 7.07 or 163.3 ± 9.22). The activated MAPK declined to control level after 20 min in these cells. In contrast, treatment with FSH significantly activated MAPK after 5 min and sustained for 60 min in IOSE-29EC cells (Figs. 46A and B, 100.0 ± 9.35 % *vs.* 195.0 ± 10.70, 184.6 ± 14.47 or 190.8 ± 14.26). FSH-stimulated MAPK activation was completely abolished by pretreatment with PD98059 in both cell lines. In addition, treatment with PD98059 alone significantly decreased MAPK activity as well in both IOSE-29 and IOSE-29EC cells (Figs. 45B and 46B).

4.5. Effects of FSH and/or PKC inhibitor staurosporin on MAPK activation

To assess whether the PKC signal transduction pathway is involved in MAPK activation in neoplastic OSE cells, the cells were treated with FSH (100 ng/ml) and/or pretreated with staurosporin (1 μM), a PKC inhibitor, in a time dependent manner. As demonstrated in Figs. 47A and B, treatments with FSH resulted in a significant increase in MAPK activation at 5-10 min in IOSE-29 cells as expected. Similarly, treatments with FSH induced a significant increase of MAPK activation after 5 min and sustained for 20 min in IOSE-29EC cells (Figs. 48A and B). FSH-stimulated MAPK activation was completely abolished by pretreatment with staurosporin for 30 min in both cell lines. Treatment with staurosporin alone resulted in a significant decrease of P-MAPK activation as well in both IOSE-29 and IOSE-29EC cells (Figs. 47B and 48B).

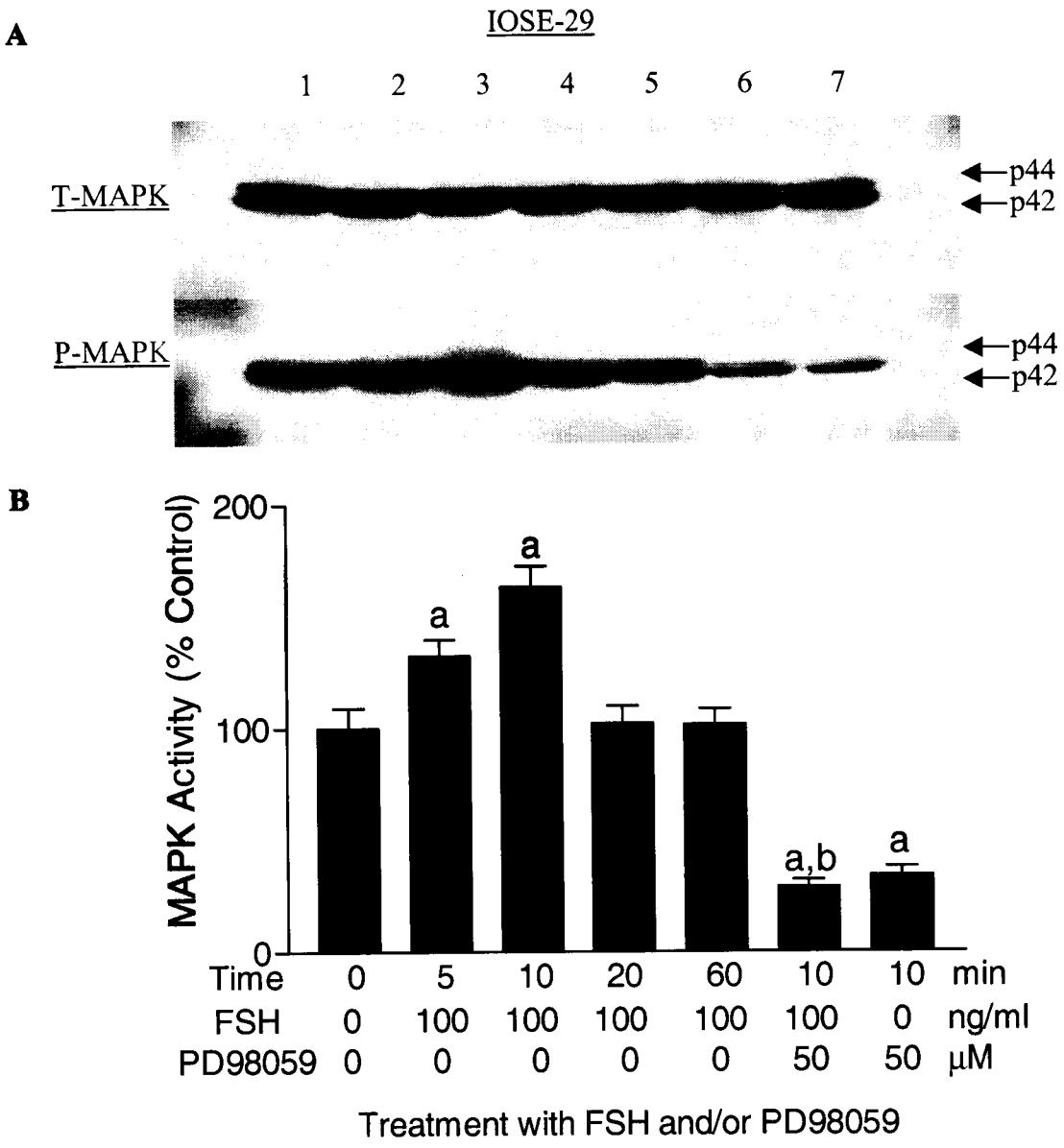


Figure 45. Effects of FSH in the presence or absence of PD98059 on MAPK activation in IOSE-29 cells. A time-dependent experiment was performed following treatment with FSH (100 ng/ml) and/or pretreatment with PD98059 (50 μM) on MAPK activity. The P-MAPK normalized by T-MAPK was analyzed in IOSE-29 (A and B). Data are shown as the means of three individual experiments, and are presented as the mean ± SD. a, $P<0.05$ *vs.* untreated control; b, $P<0.05$ *vs.* FSH (100 ng/ml) treatment for 10 min. 1, untreated control; 2, FSH (100 ng/ml) treatment for 5 min; 3, FSH (100 ng/ml) treatment for 10 min; 4, FSH (100 ng/ml) treatment for 20 min; 5, FSH (100 ng/ml) treatment for 60 min; 6, FSH (100 ng/ml) plus PD98059 (50 μM) treatment for 10 min; 7, PD98059 (50 μM) treatment.

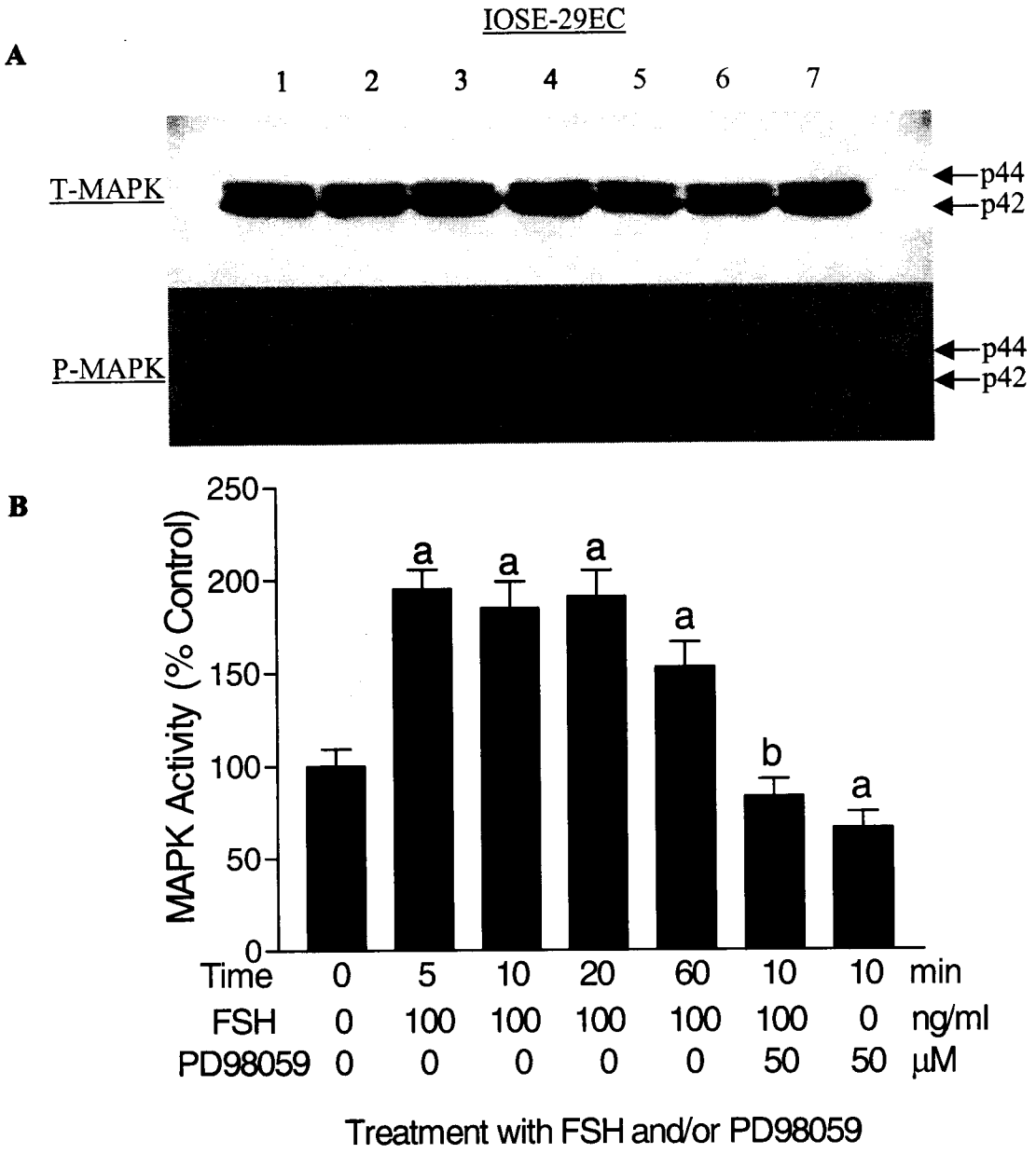


Figure 46. Effects of FSH in the presence or absence of PD98059 on MAPK activation in IOSE-29EC cells. A time-dependent experiment was performed following treatment with FSH (100 ng/ml) and/or pretreatment with PD98059 (50 μM) on MAPK activity. The P-MAPK normalized by T-MAPK was analyzed in IOSE-29EC (A and B). Data are shown as the means of three individual experiments, and are presented as the mean ± SD. a, $P<0.05$ *vs.* untreated control; b, $P<0.05$ *vs.* FSH (100 ng/ml) treatment for 10 min. 1, untreated control; 2, FSH (100 ng/ml) treatment for 5 min; 3, FSH (100 ng/ml) treatment for 10 min; 4, FSH (100 ng/ml) treatment for 20 min; 5, FSH (100 ng/ml) treatment for 60 min; 6, FSH (100 ng/ml) plus PD98059 (50 μM) treatment for 10 min; 7, PD98059 (50 μM) treatment.

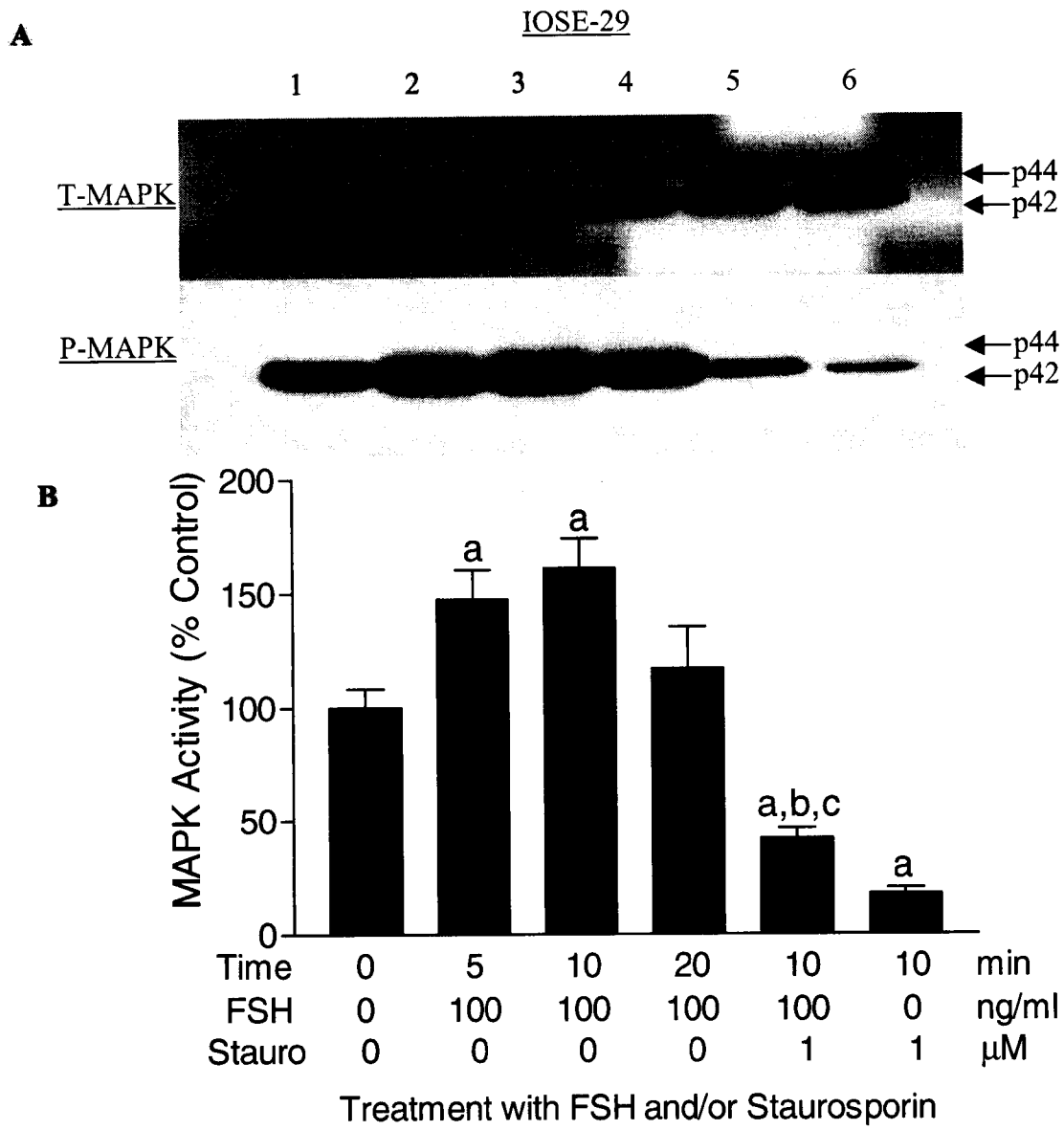


Figure 47. Effects of FSH in the presence or absence of staurosporin on MAPK activation in IOSE-29 cells. To assess whether the PKC signal transduction pathway is involved in MAPK activation in neoplastic OSE cells, the cells were treated with FSH (100 ng/ml) and/or pretreated with staurosporin (1 μM), a PKC inhibitor, in a time dependent manner. The P-MAPK normalized by T-MAPK was analyzed in IOSE-29 (A and B). Data are shown as the means of three individual experiments, and are presented as the mean ± SD. a, $P<0.05$ *vs.* untreated control; b, $P<0.05$ *vs.* FSH (100 ng/ml) treatment for 10 min; c, $P<0.05$ *vs.* staurosporin (1 μM) treatment. 1, untreated control; 2, FSH (100 ng/ml) treatment for 5 min; 3, FSH (100 ng/ml) treatment for 10 min; 4, FSH (100 ng/ml) treatment for 20 min; 5, FSH (100 ng/ml) plus staurosporin (1 μM) treatment for 10 min; 6, Staurosporin (1 μM) treatment.

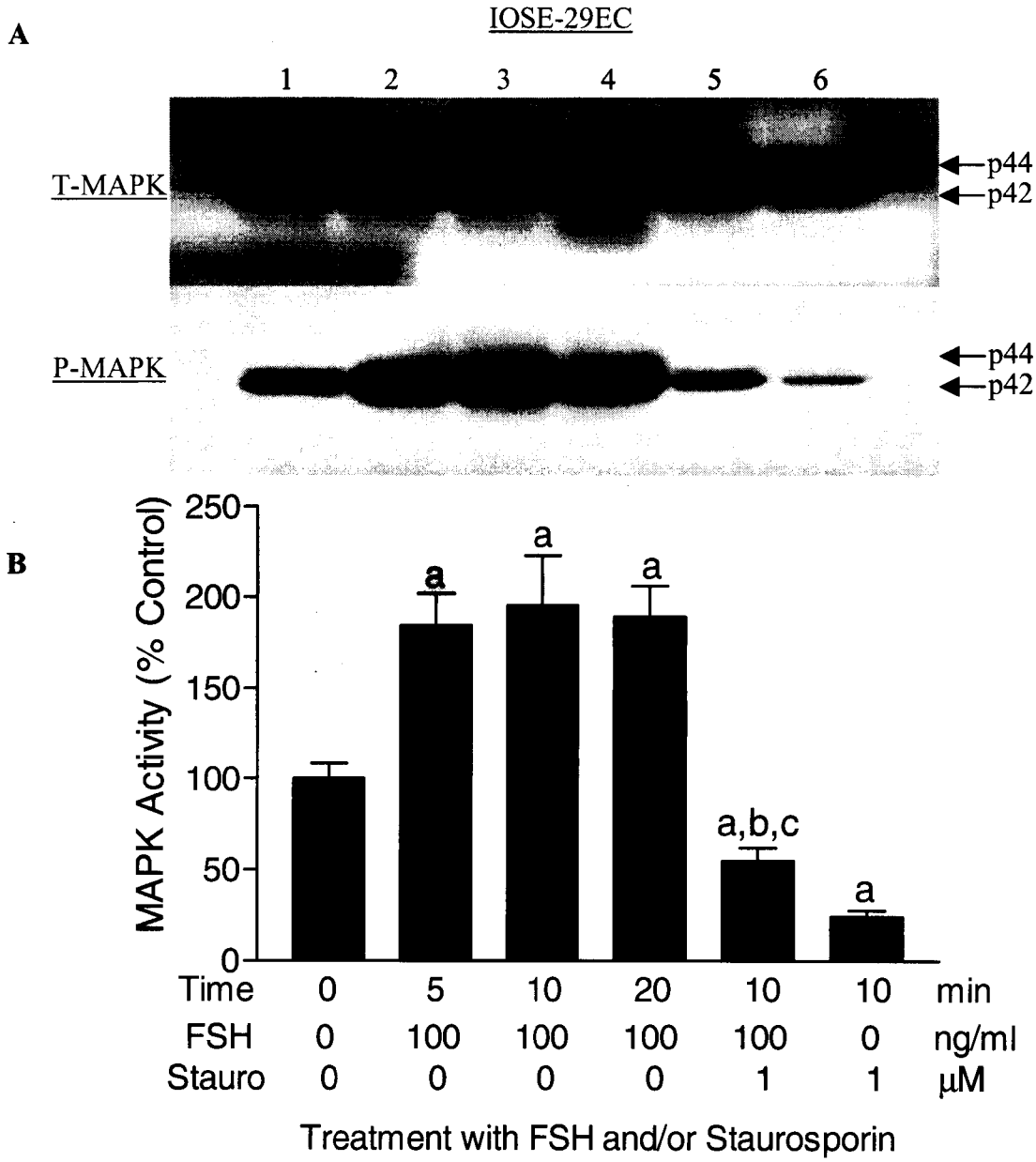


Figure 48. Effects of FSH in the presence or absence of staurosporin on MAPK activation in IOSE-29EC cells. To assess whether the PKC signal transduction pathway is involved in MAPK activation in neoplastic OSE cells, the cells were treated with FSH (100 ng/ml) and/or pretreated with staurosporin (1 μM), a PKC inhibitor, in a time dependent manner. The P-MAPK normalized by T-MAPK was analyzed in IOSE-29EC (A and B). Data are shown as the means of three individual experiments, and are presented as the mean ± SD. a, $P<0.05$ *vs.* untreated control; b, $P<0.05$ *vs.* FSH (100 ng/ml) treatment for 10 min; c, $P<0.05$ *vs.* staurosporin (1 μM) treatment. 1, untreated control; 2, FSH (100 ng/ml) treatment for 5 min; 3, FSH (100 ng/ml) treatment for 10 min; 4, FSH (100 ng/ml) treatment for 20 min; 5, FSH (100 ng/ml) plus staurosporin (1 μM) treatment for 10 min; 6, Staurosporin (1 μM) treatment.

4.6. Effects of FSH and/or PD98059, staurosporin on MAPK activation

To examine the role of FSH on MAPKs in OVCAR-3 and SKOV-3 cells, the cells were pretreated with 50 μM PD98059 or 1 μM staurosporin for 30 min, followed by treatment with 100 ng/ml FSH for 10 min. As shown in Fig. 49A, treatment with FSH appeared to induce a significant increase in P-MAPK activation in OVCAR-3 cells. In contrast, no difference was observed in SKOV-3 cells following FSH treatment (Fig. 49B). Pre-treatments of PD98059 or staurosporin resulted in a decrease of FSH-induced P-MAPK activation in OVCAR-3 cells (Fig. 49A), whereas it appears that pre-treatments with PD98059 or staurosporin did not affect on MAPK activity in SKOV-3 cells (Fig. 49B).

4.7. Effect of FSH and/or PD98059 on Elk-1 phosphorylation

The Ets family transcription factor, Elk-1 is a physiological substrate for p42 MAPK and p44 MAPK (Gille *et al.*, 1995; Janknecht *et al.*, 1993). To investigate whether the FSH-induced activation of MAPK leads to phosphorylation of Elk-1 *in vitro*, the cells were treated with FSH (100 ng/ml) for 10 min and/or PD98059 (50 μM) for 30 min. As shown in Fig. 50, treatment of FSH resulted in a significant increase in Elk-1 phosphorylation, whereas pretreatment with PD98059 significantly inhibited FSH-induced Elk-1 phosphorylation in both IOSE-29 (100.0 ± 9.66 % *vs.* 173.5 ± 14.59 or 52.0 ± 6.27) and IOSE-29EC cells (100.0 ± 10.46 % *vs.* 196.3 ± 13.76 or 56.1 ± 7.70).

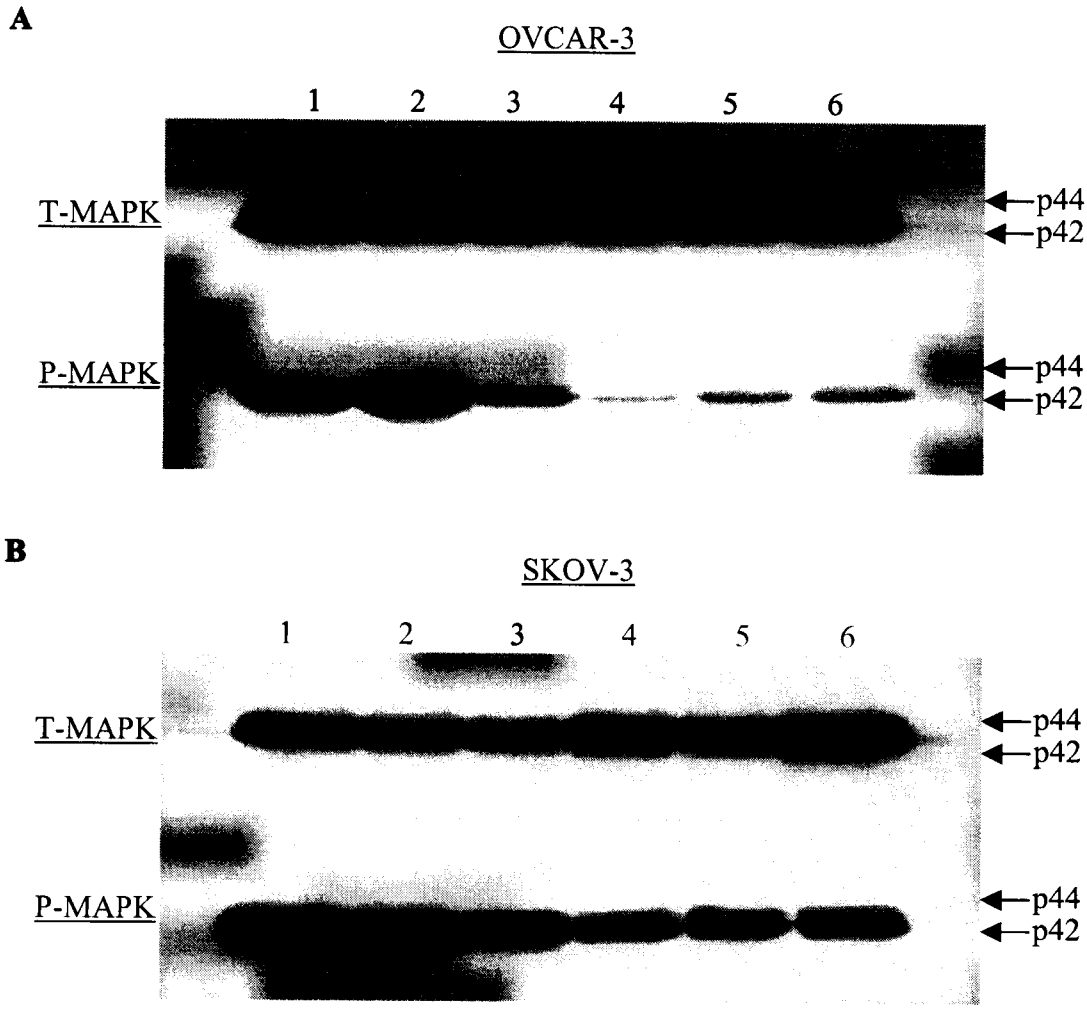


Figure 49. Effects of FSH in the presence or absence of PD98059 or staurosporin on MAPK activation in ovarian cancer cell lines. To examine the role of FSH on MAPKs in OVCAR-3 and SKOV-3 cells, the cells were pretreated with 50 μM PD98059 or 1 μM staurosporin for 30 min, followed by treatment with 100 ng/ml FSH for 10 min. The P-MAPK normalized by T-MAPK was analyzed in OVCAR-3 (A) and SKOV-3 cells (B). 1, untreated control; 2, FSH (100 ng/ml) treatment; 3, FSH (100 ng/ml) plus PD98059 (50 μM) treatment; 4, PD98059 (50 μM) treatment; 5, FSH (100 ng/ml) plus staurosporin (1 μM) treatment; 6, Staurosporin (1 μM) treatment.

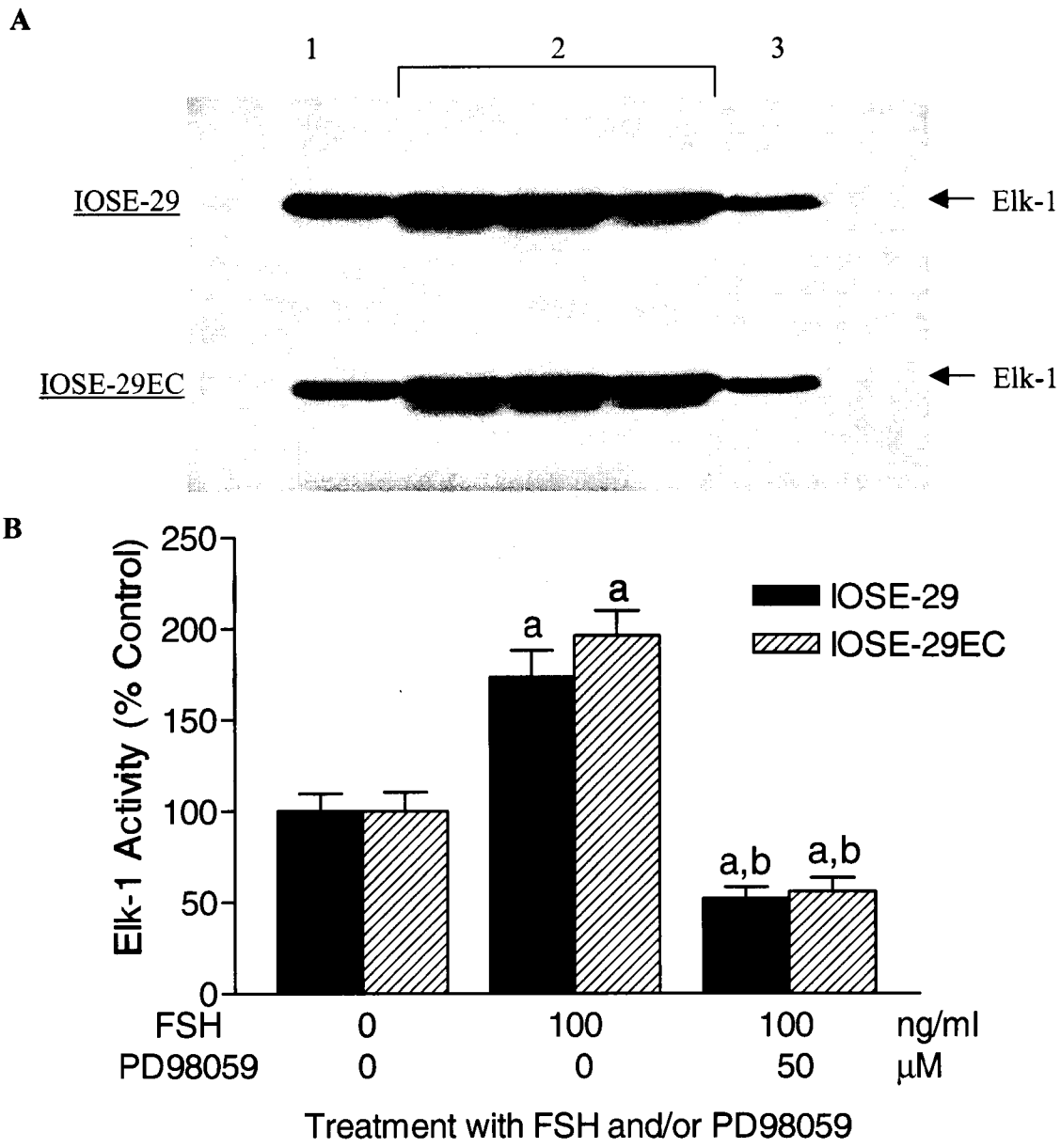


Figure 50. Effect of FSH in the presence or absence of PD98059 on Elk-1 phosphorylation. To investigate whether the FSH-induced activation of MAPK leads to phosphorylation of Elk-1 *in vitro*, the cells were treated with FSH (100 ng/ml) for 10 min and/or PD98059 (50 μM) for 30 min. The phosphorylation of Elk-1 was investigated following FSH and/or PD98059 treatment in IOSE-29 and IOSE-29EC cells (A and B). Data are shown as the means of three individual experiments, and are presented as the mean ± SD. a, $P<0.05$ *vs.* untreated control; b, $P<0.05$ *vs.* FSH (100 ng/ml) treatment for 10 min. 1, untreated control; 2, FSH (100 ng/ml) treatment for 10 min; 3, FSH (100 ng/ml) plus PD98059 (50 μM) treatment.

4.8. Effects of MAPK and PKC inhibitors on FSH-stimulated cell growth

To evaluate the effect of MAPK and PKC inhibitors on FSH-stimulated cell growth, the cells were pretreated with PD98059 (50 μM) or staurosporin (1 μM) for 30 min and then treated with FSH (100 ng/ml) for 24 h in IOSE-29 and IOSE-29EC cells. A [^{3}H]thymidine incorporation assay was performed as previously described in *the Materials and Methods*. Treatment with FSH (100 ng/ml) resulted in a significant growth-stimulation in these cells as expected (Fig. 51). As seen in Fig. 51, pre-treatments with PD98059 and staurosporin attenuated completely FSH-stimulated cell growth in both IOSE-29 and IOSE-29EC cells.

4.9. Effects of FSH on intracellular cAMP accumulation

To investigate whether FSH modulates intracellular cAMP levels, the cells were treated with FSH (100 ng/ml), and intracellular cAMP levels were measured. Treatment of FSH did not affect basal intracellular cAMP levels in IOSE-29 and IOSE-29EC cells (Fig. 52), whereas FSH treatment induced a significant increase of intracellular cAMP in human granulosa luteal cells (hGLCs).

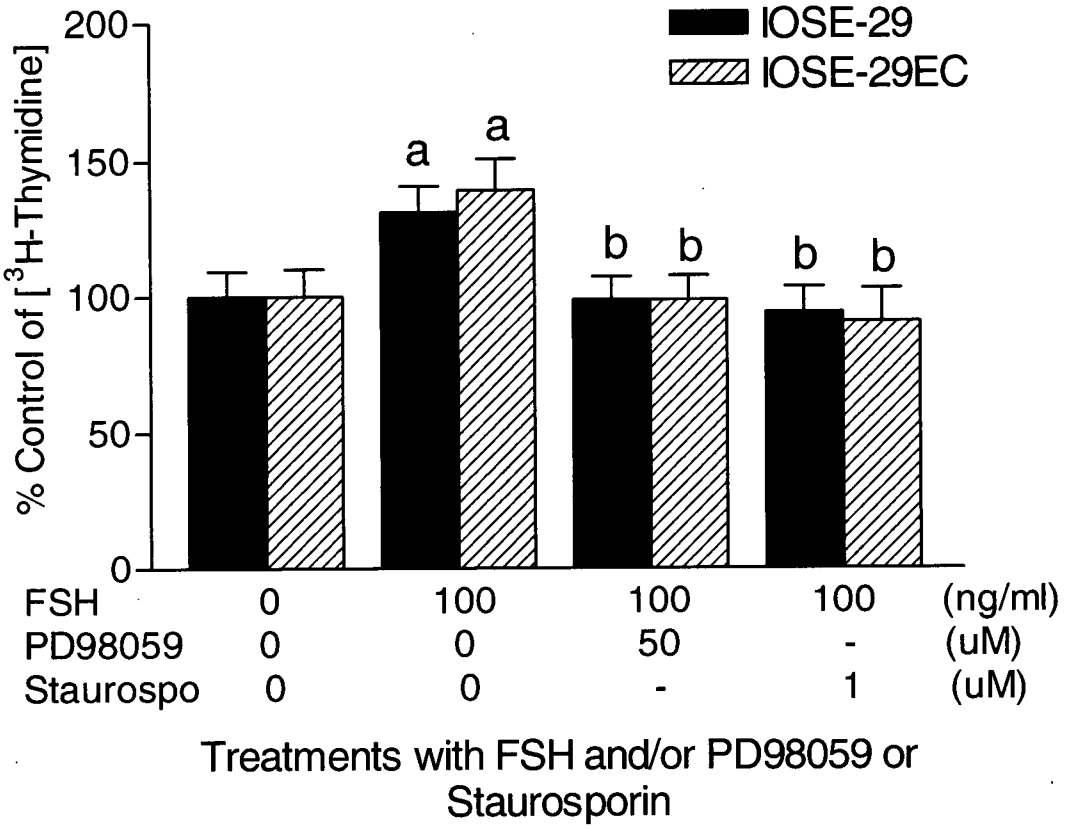


Figure 51. Effects of MAPK and PKC inhibitors on FSH-stimulated cell growth. To evaluate the effect of MAPK and PKC inhibitors on FSH-stimulated cell growth, the cells were pretreated with PD98059 (50 μM) or staurosporin (1 μM) for 30 min and then treated with FSH (100 ng/ml) for 24 h in IOSE-29 and IOSE-29EC cells. A [^{3}H]thymidine incorporation assay was performed as previously described in *the Materials and Methods*. Data are shown as the means of three individual experiments, and are presented as the mean ± SD. a, $P<0.05$ *vs.* untreated control; b, $P<0.05$ *vs.* FSH (100 ng/ml) treatment.

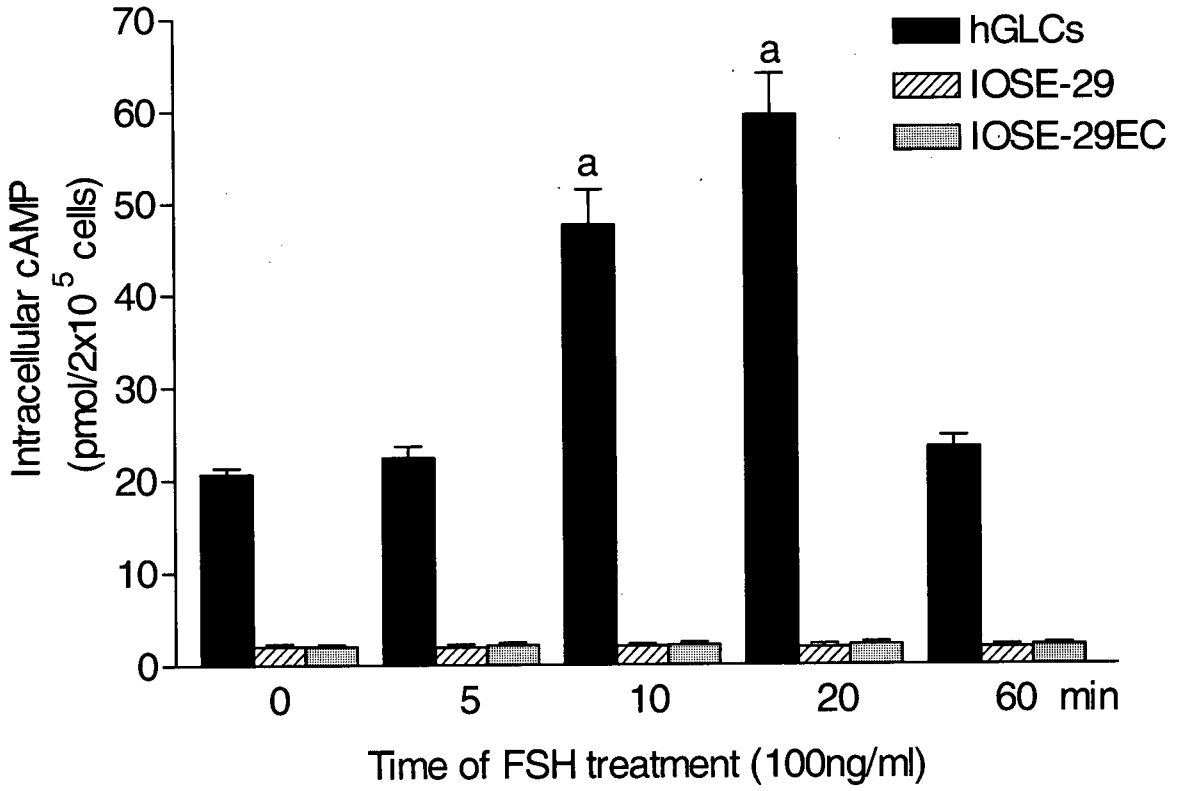


Figure 52. Effect of FSH on intracellular cAMP accumulation. To investigate whether FSH modulates intracellular cAMP levels, the cells were treated with FSH (100 ng/ml), and intracellular cAMP levels were measured in IOSE-29, IOSE-29EC and human granulose luteal cells (hGLCs). Data are shown as the means of three individual experiments, and are presented as the mean ± SD. a, $P<0.05$ *vs.* untreated control.

5. *EXPERIMENT E*

5.1. Expression of GnRH-II mRNA

The mRNA expression of GnRH-II in normal and neoplastic OSE cells was investigated as previously described. A predicted PCR product of GnRH-II was obtained as 327-bp using specific primers and confirmed by Southern blot analysis using DIG-labeled probes in normal OSE and IOSE cell lines (Fig. 53A) and sequence analysis (data not shown). Similarly, a PCR product of GnRH-II was also detected in primary cultured ovarian cancers from the patients (PC-OVC) and ovarian cancer cell lines (CaOV-3, OVCAR-3, and SKOV-3) as shown in Fig. 53B. These results indicate that GnRH-II mRNAs are expressed in both normal and neoplastic OSE cells.

5.2. Expression of GnRH-R mRNA

The mRNA expression of GnRH-R in IOSE cells was investigated by RT-PCR and Southern blot analysis. A predicted PCR product of GnRH-R was obtained as 347-bp and confirmed by Southern blot analysis using DIG-labeled probes (Fig. 54) and sequence analysis (data not shown). OVCAR-3 cells were used for positive control. As demonstrated in Fig. 54, the GnRH-R mRNAs are expressed in IOSE cell lines (IOSE-29, IOSE-29EC, IOSE-29EC/T4 and IOSE-29EC/T5).

5.3. Effects of GnRH-I and -II on proliferative index

To evaluate the role of GnRH-I and -II in IOSE cell lines, the cells were treated with increasing concentrations (10^{-9}– 10^{-7} M) of GnRH-I and GnRH-II for 6 days and a [^{3}H]thymidine

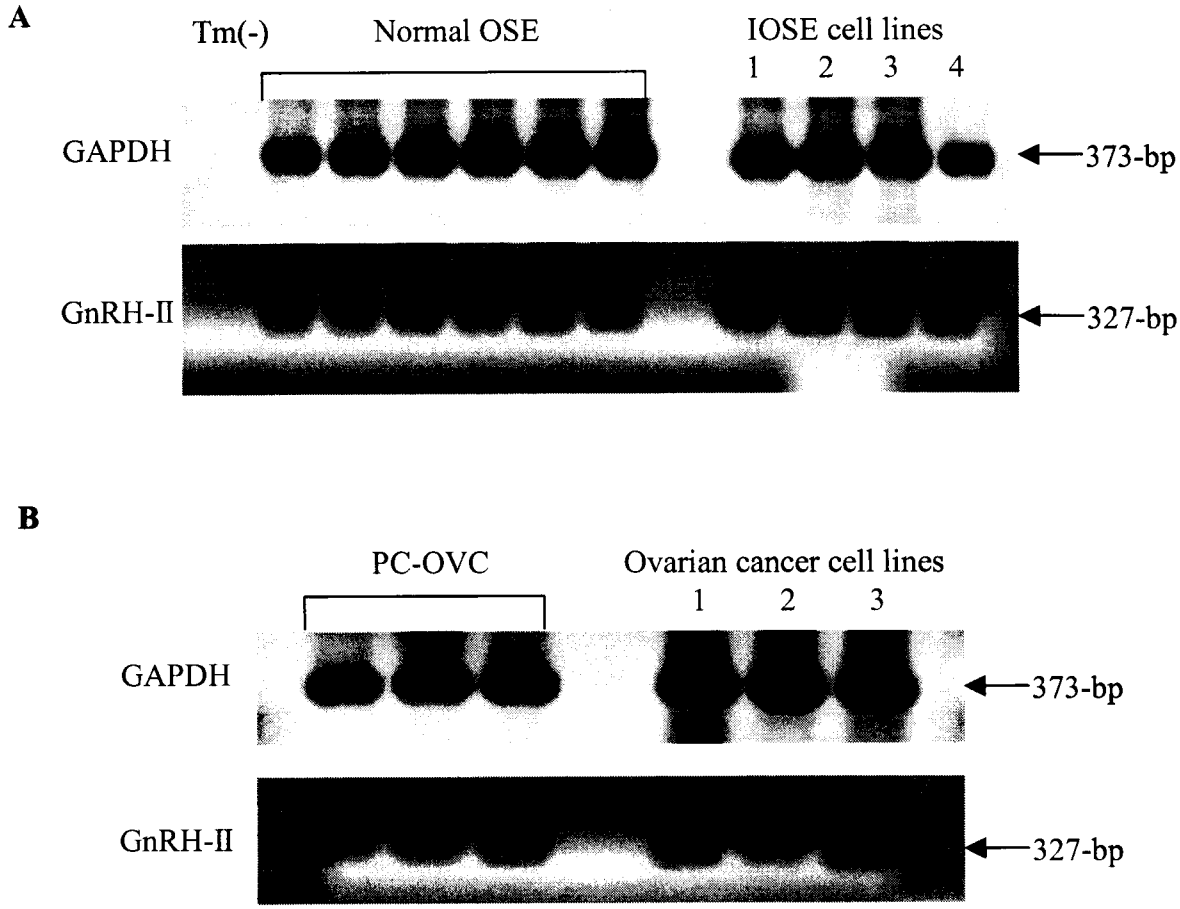


Figure 53. Expression of GnRH-II mRNA in normal and neoplastic OSE cells. The mRNA expression of GnRH-II in normal and neoplastic OSE cells was investigated as previously described. A predicted PCR product of GnRH-II was obtained as 327-bp using specific primers and confirmed by Southern blot analysis using DIG-labeled probes in normal OSE and IOSE cell lines (1: IOSE-29; 2: IOSE-29EC; 3: IOSE-29EC/T4; 4: IOSE-29EC/T5). Similarly, a PCR product of GnRH-II was also detected in primary cultured ovarian cancers from the patients (PC-OVC) and ovarian cancer cell lines (1: CaOV-3; 2: OVCAR-3; 3: SKOV-3).

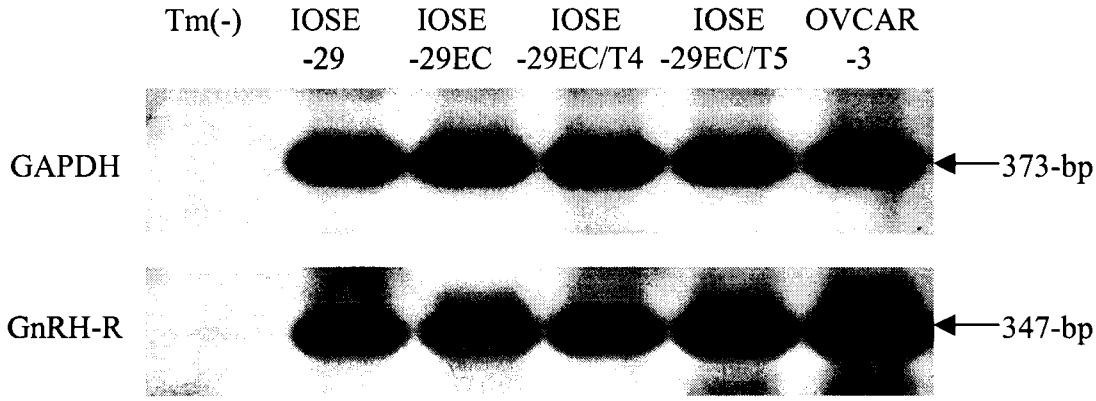


Figure 54. Expression of GnRH-R mRNA in IOSE cell lines and OVCAR-3 cells. The mRNA expression of GnRH-R in IOSE cells was investigated by RT-PCR and Southern blot analysis. A predicted PCR product of GnRH-R was obtained as 347-bp and confirmed by Southern blot analysis using DIG-labeled probes. OVCAR-3 cells were used for positive control.

incorporation assay was performed as previously described (Kang *et al.*, 2000). Treatments with increasing doses of GnRH-I resulted in a significant growth-inhibition in both IOSE-29 and IOSE-29EC cells (Fig. 55). Co-treatment with antide plus GnRH-I completely blocked the growth-inhibitory effect of GnRH-I in both cell lines. In addition, treatments with same concentrations of GnRH-II also induced a significant growth-inhibition of IOSE-29 and IOSE-29EC cells (Fig. 56). Similarly, co-treatment with antide plus GnRH-II completely blocked the growth-inhibitory effect of GnRH-II in both cell lines. These results indicate that neoplastic OSE cells are responsive to GnRH-I and -II treatments, which resulted in a growth-inhibition through GnRH-R in these cells.

5.4. Effects of GnRH-I and –II on apoptosis

To examine the role of GnRH-I and –II in the induction of apoptosis, DNA fragmentation was measured by cell death detection ELISA. To quantify the induction of apoptosis, IOSE-29 and IOSE-29EC cells were treated with GnRH-I and –II for 6 days. As shown in Fig 57A, treatments with GnRH-I (D-Ala-GnRH, 10^{-7} M) increased DNA fragmentation in both cell lines. Co-treatment with antide (10^{-7} M) with GnRH-I blocked completely GnRH-I effect on the induction of DNA fragmentation. In addition, treatments with GnRH-II (10^{-7} M) resulted in an increase of DNA fragmentation in IOSE-29 and IOSE-29EC cells (Fig. 57B). Similarly, co-treatment with antide (10^{-7} M) with GnRH-II blocked completely GnRH-II effect on the induction of DNA fragmentation.

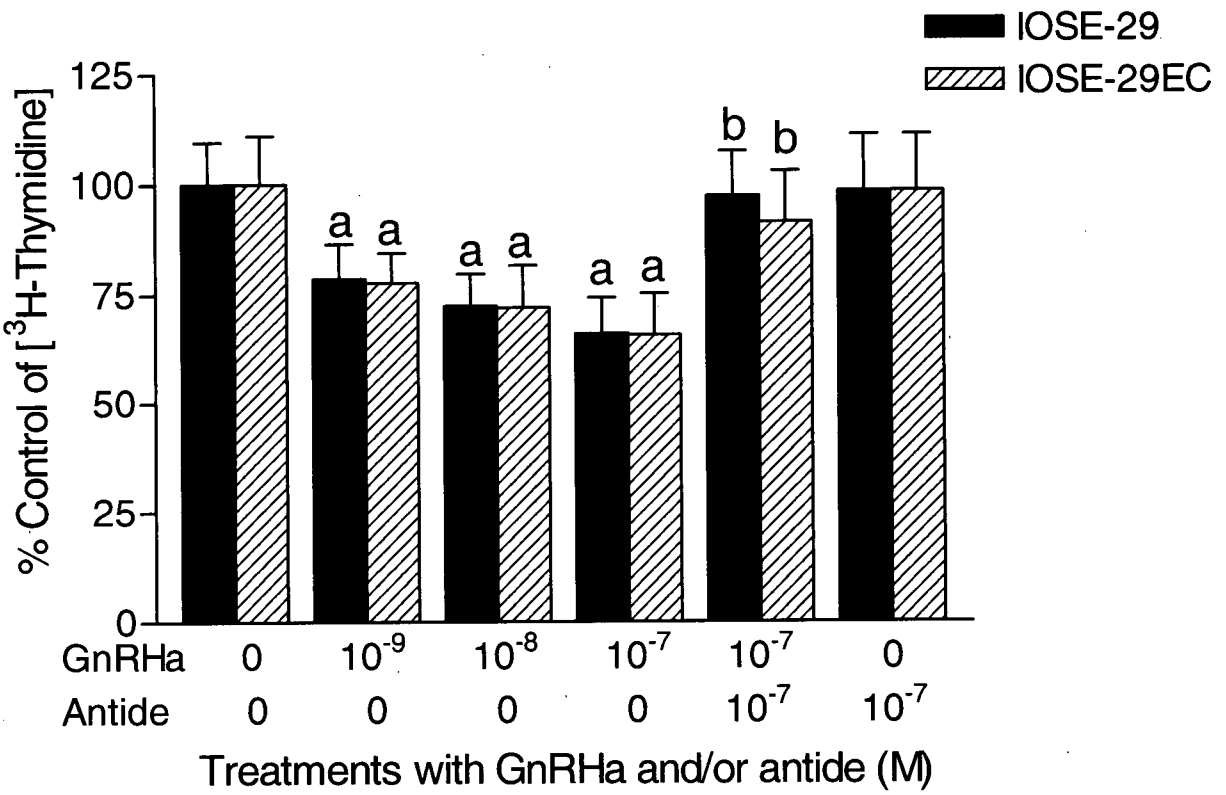


Figure 55. Effects of GnRH-I on proliferative index in IOSE cell lines. To evaluate the role of GnRH-I in IOSE cell lines, the cells were treated with increasing concentrations (10^{-9} – 10^{-7} M) of GnRH-I for 6 days and a [^{3}H]thymidine incorporation assay was performed as previously described (Kang *et al.*, 2000). Treatments with increasing doses of GnRH-I resulted in a significant growth-inhibition in both IOSE-29 and IOSE-29EC cells. Co-treatment with antide plus GnRH-I completely blocked the growth-inhibitory effect of GnRH-I in both cell lines.

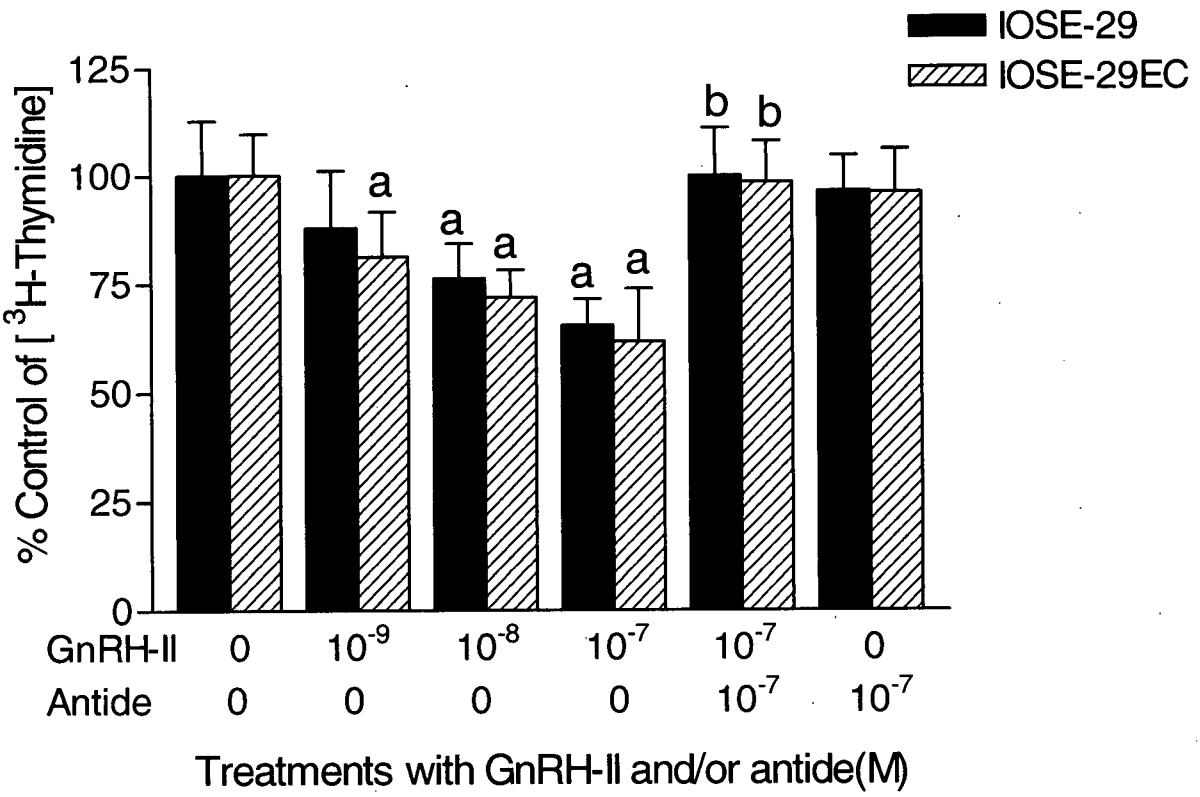


Figure 56. Effects of GnRH-II on proliferative index in IOSE cell lines. To evaluate the role of GnRH-II in IOSE cell lines, the cells were treated with increasing concentrations (10^{-9} – 10^{-7} M) of GnRH-II for 6 days and a [^{3}H]thymidine incorporation assay was performed as previously described (Kang *et al.*, 2000). Treatments with increasing doses of GnRH-II resulted in a significant growth-inhibition in both IOSE-29 and IOSE-29EC cells. Co-treatment with antide plus GnRH-II completely blocked the growth-inhibitory effect of GnRH-II in both cell lines.

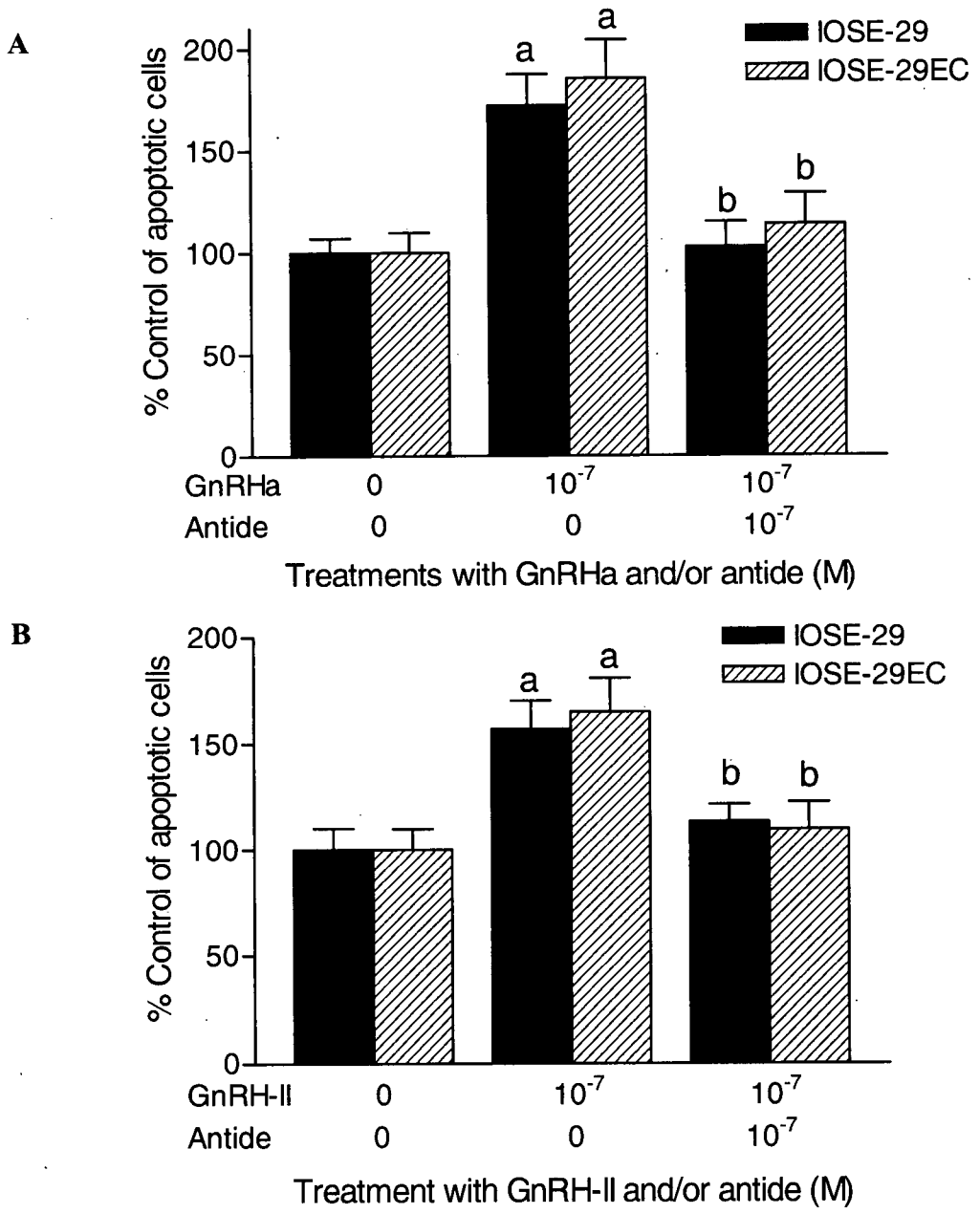


Figure 57. Effects of GnRH-I and –II on apoptosis. To examine the role of GnRH-I and –II in the induction of apoptosis, DNA fragmentation was measured by cell death detection ELISA. To quantify the induction of apoptosis, IOSE-29 and IOSE-29EC cells were treated with GnRH-I and –II for 6 days. Treatments with GnRH-I (D-Ala-GnRH, 10^{-7} M) and GnRH-II (10^{-7} M) resulted in an increase of DNA fragmentation in IOSE-29 and IOSE-29EC cells. Co-treatment with antide (10^{-7} M) with GnRH-I and -II blocked completely GnRH-I and -II effect on the induction of DNA fragmentation.

5.5. Effects of GnRH-I and –II on the regulation of bax and bcl-2 proteins

To investigate the mechanism of GnRH-I and -II in the induction of apoptosis, the regulation of apoptotic bax and bcl-2 was examined by immunoblot analysis. The IOSE-29 and IOSE-29EC cells were treated with increasing doses (10^{-9} to 10^{-7} M) of GnRH-I and GnRH-II respectively for 24 h and immunoblot analysis was performed as described in *the Material and Methods*. Bax and bcl-2 protein were detected at 21 kDa and 26 kDa respectively. As seen in Fig 58, treatments with GnRH-I had no effect on both bax and bcl-2 proteins in both cell lines. In addition, no significant difference of bax and bcl-2 proteins was observed in GnRH-II treatments (Fig. 58). The loaded amount of proteins in treatment groups was normalized by actin protein (41 kDa, data not shown).

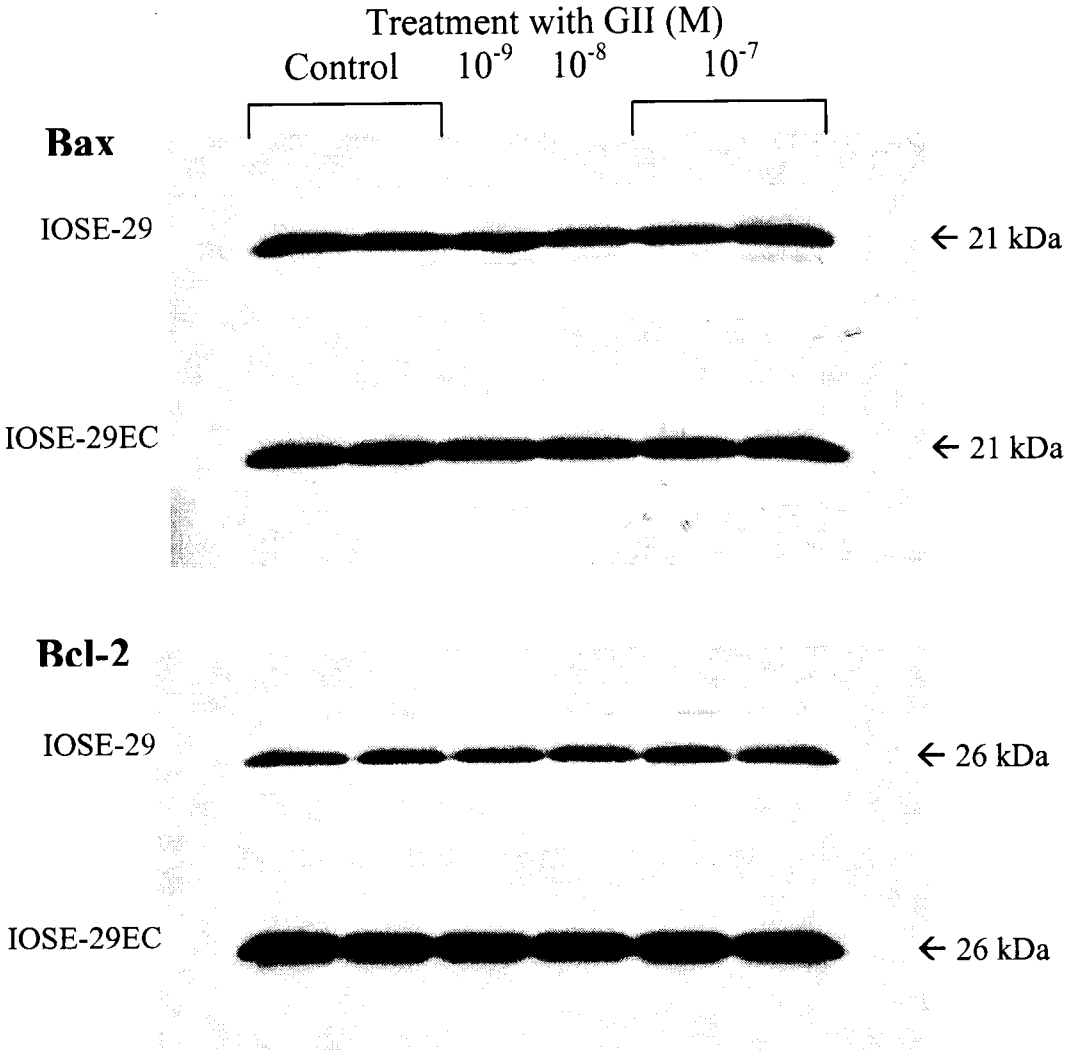


Figure 58. Effects of GnRH-I and –II on the regulation of bax and bcl-2 proteins. To investigate the mechanism of GnRH-I and -II in the induction of apoptosis, the regulation of apoptotic bax and bcl-2 was examined by immunoblot analysis. The IOSE-29 and IOSE-29EC cells were treated with increasing doses (10^{-9} to 10^{-7} M) of GnRH-I and GnRH-II respectively for 24 h and immunoblot analysis was performed as described in the Material and Methods. Bax and bcl-2 protein were detected at 21 kDa and 26 kDa respectively. The loaded amount of proteins in treatment groups was normalized by actin protein (41 kDa, data not shown).

IV. DISCUSSION

1. *Experiment A*

Normal OSE and OVCAR-3 cells have been demonstrated to express all forms of inhibin subunits and activin receptors at the mRNA and protein levels (Fukuda *et al.*, 1998; Ito *et al.*, 2000, Welt *et al.*, 1997). In addition, OVCAR-3 cells expressed the Smad-4 mRNAs and Smad-2, which are proteins specific for TGF-β signaling pathway (Ito *et al.*, 2000). The present study clearly demonstrates that activin/inhibin subunits and activin receptor IIB are differentially expressed in normal and neoplastic OSE cells. The α and βA subunit were highly expressed in normal OSE when compared to OVCAR-3 cells. In contrast, the mRNA level of βB subunit was greatly expressed in OVCAR-3 cells when compared to normal OSE cells. These results indicate that inhibin may be predominantly expressed in this ovarian compartment. In contrast, OVCAR-3 cells expressed a low level of the α subunit, but a high level of βB subunit, when compared to normal OSE cells, suggesting that activin may be predominantly expressed in this cell line. These results are in an agreement with the recent finding that inhibin α subunit expression was found in 47% of OSE, but was not found in the epithelial component of ovarian cystadenomas, tumors of low malignant potential (LMP), or carcinomas by immunohistochemisty and RT-PCR. βA subunit was expressed in 93% of OSE, in the epithelial component of all cystadenomas, in 81% of LMP tumors and in 72% of carcinomas, suggesting that imbalanced expression of inhibin and activin subunits in OSE may represent an early event that leads to epithelial proliferation (Zheng *et al.*, 1998). The β subunits may be dominantly expressed in neoplastic OSE cells because most epithelial ovarian tumors (96%) synthesize and secrete activin *in vitro* (Welt *et al.*, 1997). The detection of βA subunit alone in the absence of α subunit suggests the

synthesis of activin with little or no inhibin expression in epithelial ovarian cancer (Zheng *et al.*, 1998). The switch from an inhibin dominant to an activin dominant status in neoplastic OSE may play a role in epithelial ovarian tumorigenesis. Although no difference was observed in the basal mRNA level of activin receptor IIA, OVCAR-3 cells expressed a higher level of activin receptor IIB, indicating that this cell line may be more responsive to activin treatment than normal OSE cells. Our observation that OVCAR-3 expressed low level of α subunit appears to be at variance somewhat with another report (Di Simone *et al.*, 1996) that α subunit was not expressed in six other ovarian cancer cell lines. The apparent discrepancy between our results and the study by Di Simone *et al.* may be due to 1) different cell lines used, 2) the different specificity of primers used in the experiments, 3) the different sensitivity of Southern blot analysis using [^{32}P]ATP-labeled oligonucleotide probes or digoxigenine-labeled cDNA probes, 4) different culture conditions.

Most primary epithelial ovarian tumors (96%) synthesize and secrete activin *in vitro* and serum levels of activin are frequently elevated in women with epithelial ovarian cancer (Welt *et al.*, 1997). In the present study, activin stimulated cell proliferation in a neoplastic OSE cell line, OVCAR-3, but not in normal OSE cells, whereas the stimulatory effect of activin was attenuated by follistatin treatment. These results indicate that activin is mediated *via* its receptors, by stimulating the proliferative index of OVCAR-3. We have previously shown that OVCAR-3 cells expressed activin receptors, supporting the notion that activin may act in an autocrine manner (Fukuda *et al.*, 1998). Treatments with activin (1-100 ng/ml) resulted in an increase, whereas follistatin treatment (1-100 ng/ml) resulted in a decrease in cellular proliferation of CaOV-3, CaOV-4, and SW-626 ovarian cancer cell lines (Di Simone *et al.*, 1996). The proliferative index of normal OSE cells in the present study was unaffected following activin

treatment, even though these cells also express activin receptors (Welt *et al.*, 1997). Therefore, it is hypothesized that activin may stimulate the proliferation of ovarian tumors following the transformation of normal OSE to neoplastic OSE cells.

To determine if the growth stimulatory effect of activin A is mediated via an autocrine mechanism in neoplastic OSE, the expression of activin/inhibin subunits and activin receptors by rh-activin A was examined in OVCAR-3 cells. Treatment with activin resulted in a significant increase in βA subunit mRNA levels in a dose- and time-dependent manner. In addition, this stimulatory effect of activin on βA subunit mRNA expression was attenuated following treatment with follistatin, suggesting that exogenous activin A may enhance activin A expression through its receptors. Thus, activin A may enhance cellular proliferation *via* an autocrine pathway in ovarian carcinoma cells. Similarly, activin A induced an increase in the α subunit mRNA in OVCAR-3, implicating an increase in inhibin expression (LaPolt *et al.*, 1989). In addition, biologically active follistatin, capable of binding human activin, has been demonstrated to secrete in an ovarian teratocarcinoma cell line (PA-1) (Wang *et al.*, 1996). Treatment with activin induced up to a 12-fold increase in βA subunit expression and up to a 2-fold increase in α subunit. These results indicate that exogenous activin A may induce more activin A than inhibin A production in OVCAR-3 cells, further suggesting an autocrine role of activin in ovarian cancer growth. Although the βB subunit is highly expressed in OVCAR-3 cells, it appears that the primary targets for activin treatment are α and βA subunit but not βB subunit in OVCAR-3 cells. Our results on the effects of activin on activin/inhibin subunit mRNA levels in human neoplastic OSE (i.e., OVCAR-3) cells are in an agreement with the previous findings in the rat ovary. In rat granulosa cells, activin induced the expression of both α and βA subunit mRNAs, but no difference in βB subunit level was observed in these cells (LaPolt *et al.*, 1989). In contrast,

activin A induced only βB subunit mRNA in a dose- and time-dependent manner without affecting basal expression levels of the α and βA subunit mRNAs in human granulosa cells (Eramaa *et al.*, 1995). Together, these results indicate that activin/inhibin subunits may be differentially regulated by activin in an autocrine manner. In this regard, activin/inhibin subunits may be differentially regulated by other endocrine and autocrine factors as well. TGF-β has been found to regulate βB subunit mRNA level in cultured human granulosa-luteal cells, whereas gonadotropins potently induce the α- and βA subunit mRNAs, suggesting that distinct components of the activin/inhibin system are regulated (Eramaa and Ritvos, 1996). In addition, estrogen may affect expression of the activin/inhibin subunits in the rat granulosa cells, and regulate the production of inhibin and activin differentially (Tate *et al.*, 1996). The activation of protein kinase-A and -C by 8-bromo-cAMP and 12-*O*-tetradecanoylphorbol 13-acetate (TPA), respectively, results in imbalanced expression of activin/inhibin subunits in human granulosa-luteal (Tuuri *et al.*, 1996) and placental cells (Li *et al.*, 1994) *in vitro*.

Multiple activin receptors are present in the human ovary and placenta, suggesting activin may play a role in these tissues (Sidis *et al.*, 1998; Peng *et al.*, 1999). Normal and neoplastic OSE cells have been demonstrated to express all known subtypes of activin receptors, further supporting a role for activin in ovarian neoplastic progression. (Welt *et al.*, 1997). Activin may act as an autocrine growth factor in stimulating the proliferation of gonadal tumor cell lines derived from inhibin-α and p53-deficient mice (Shikone *et al.*, 1994). In addition, treatment with anti-activin A serum inhibited tumor cell replication and blocked the stimulatory action of activin on cell growth via its receptors. However, activin-responsive testicular tumor cells, derived from a $p53^{-/-}$ / α-$inhibin^{-/-}$ mouse testicular tumor, respond to activin treatment by inhibition of cell proliferation. Furthermore, in the presence of exogenous activin, mRNA levels

of activin type IIA receptor and activin/inhibin βA subunit were significantly suppressed, suggesting that the inhibition of cellular proliferation in testicular tumor cells can be induced by down-regulation of activin receptor mRNA levels (Di Simone *et al.*, 1998). However, treatment with activin, which stimulated cell proliferation, did not alter the mRNA levels of activin receptor IIA and IIB in OVCAR-3 cells.

In summary, the present study demonstrates that (1) activin/inhibin are differentially expressed in normal and neoplastic OSE (i.e., OVCAR-3) cells, (2) activin induces cell proliferation in OVCAR-3 cells, but not in normal OSE cells, and (3) activin induces an up-regulation of α and βA subunits in OVCAR-3 cells. Taken together, these results support the hypothesis that activin may be an autocrine regulator of neoplastic OSE progression.

2. *Experiment B*

Immortalized neoplastic OSE cells from human normal OSE have been generated by transfection with SV40-large T antigen and E-cadherin (Auersperg *et al.*, 1999; Maines-Bandiera *et al.*, 1992). These IOSE-29EC cells were found to be anchorage independent and formed transplantable, invasive subcutaneous and intraperitoneal adenocarcinmas in injected SCID mice (Ong *et al.*, 2000). Therefore, two additional cell lines, designated IOSE-29EC/T4 and IOSE-29EC/T5 were established from tumors that arose in IOSE-29EC-inoculated SCID mice. These cell lines may represent early neoplastic (IOSE-29), tumorigenic (IOSE-29EC) and late neoplastic (IOSE-29EC/T4 and T5) transformation stages of OSE. The characteristics of IOSE-29EC resemble those of ovarian cancer (Auersperg *et al.*, 1999; Ong *et al.*, 2000). Thus, these cell lines may be a useful *in vitro* model to study the progression of ovarian tumorigenesis.

Activin and TGF-β belong to the TGF-β superfamily, which are known to have either growth-promoting and/or growth-inhibiting activities depending on the particular cell type (Zheng *et al.*, 1998). Activin is an important regulator that mediates hormonogenesis, cellular homeostasis (cell growth and apoptosis) and differentiation (Mathews 1994). The actions of activin can be regulated by follistatin, which binds activin with high affinity and neutralizes activin actions (Woodruff, 1998). Members of this superfamily are prime candidates for the regulation of cell proliferation during morphological changes in the ovary, as well as transformation of these tissues (Mather *et al.*, 1997). In epithelial ovarian cancer, Smad-2, specific signaling pathway of the TGF-β family, was regulated by activin treatment (Schneyer *et al.*, 1994). The present study clearly demonstrated that mRNAs of activin/inhibin subunits are expressed in the IOSE cell lines. Both mRNAs and proteins for activin receptors were expressed in these cell lines, suggesting that activin may have an autocrine role in neoplastic OSE cells. Only βB subunit was expressed at lower levels in IOSE cell lines. However, no difference was observed in the expression levels of activin receptors when compared to OVCAR-3 cells. Activin/inhibin subunits are differentially expressed in normal OSE, epithelial ovarian cystadenomas, low malignant potential (LMP) tumors and ovarian carcinomas, suggesting that an imbalanced expression of activin/inhibin subunits in OSE may represent an early event that leads to epithelial proliferation (Wang *et al.*, 1996). Our recent findings have demonstrated that activin/inhibin subunits and activin receptor IIB were differentially expressed in normal and neoplastic OSE cells in the *EXPERIMENT A*. Furthermore, treatment with activin stimulated the growth of OVCAR-3 cells but not normal OSE. Thus, the differential expression and production of activin/inhibin subunits, activin receptors and follistatin suggest that activin may be involved in neoplastic OSE cell proliferation (Di Simone *et al.*, 1996). Continuous treatments of activin (1-

100 ng/ml) for 6 days resulted in a significant decrease in cell proliferation in both IOSE-29 and IOSE-29EC cell lines. These growth-inhibitory effects of activin were attenuated following co-treatment with follistatin (100 ng/ml), a specific binding protein of activin. These findings were unexpected because activin has been thought to be a growth-stimulatory factor in some ovarian cancer cell lines (Di Simone *et al.*, 1996; Fukuda *et al.*, 1998). Furthermore, most primary epithelial ovarian tumors (96%) synthesize and secrete activin *in vitro* and serum levels of activin are frequently elevated in women with epithelial ovarian cancer (Welt *et al.*, 1997). In contrast, treatment or overexpression of activin resulted in a decrease in cell proliferation, which was blocked by follistatin, in human ovarian teratocarcinoma-derived cell line (Delbaere *et al.*, 1999; McPherson *et al.*, 1997). Similarly, it has been demonstrated that activin induced growth inhibition in prostate cancer cell lines (McPherson *et al.*, 1997; Wang *et al.*, 1996b). However, no difference was observed in the proliferative index of normal OSE even though all forms of activin receptors are expressed in these cells (Di Simone *et al.*, 1996). The mechanism by which activin suppressed a growth in the IOSE cell lines remains uncertain.

Treatments with TGF-β (0.1-10 ng/ml) induced a significant decrease in the proliferative index of normal and neoplastic OSE cells in a dose-dependent manner. The expressions of TGF-β isoforms and its receptors have been demonstrated in ovarian tumors, suggesting an autocrine and/or paracrine role of TGF-β (Bartlett *et al.*, 1992; 1997; Marth *et al.*, 1990). TGF-β inhibited the proliferation of monolayers of normal human ovarian epithelial cells by 40-70% (Berchuck *et al.*, 1992) and by 95% in primary epithelial ovarian cancer cell cultures obtained directly from ascites (Hurteau *et al.*, 1994). In contrast, immortalized epithelial ovarian cancer cell lines were found to be relatively resistant to the growth inhibition by exogenous TGF-β treatment (Berchuck *et al.*, 1990; 1992). These data suggest that TGF-β may act as a growth inhibitor that

prevents inappropriate proliferation of normal OSE cells, while loss of this autocrine growth-inhibitory pathway may lead to cancer development *in vivo* and/or immortalization of cell *in vitro*. The results in this study confirm that TGF-β is a prime inhibitory regulator of cell proliferation in both normal and neoplastic ovarian cells and show that it effectively inhibits cell proliferation in early neoplastic and tumorigenic transformation stages.

Increase in proliferation and/or decrease in apoptosis play critical roles in tumorigenesis. Treatments with increasing concentrations of activin and TGF-β resulted in an increase in DNA fragmentation of IOSE-29EC cells in a dose-dependent manner. The effect of activin on induction of apoptosis was attenuated following 100 ng/ml follistatin treatment. The previous reports have demonstrated that activin has been shown to induce apoptosis in B-cell lymphoma (Koseki *et al.*, 1995; 1998), hepatoma (Chen *et al.*, 2000) and androgen-dependent prostate cancer cells (Wang *et al.*, 1996b). The exact mechanism by which TGF-β induced growth-inhibition in ovarian tumor cells remains unknown. However, previous studies suggested that binding of TGF-β to its receptors initiates a cascade of molecular events that are thought to decrease activity of cyclin-dependent kinase, resulting in arrest of cell cycle from G1 into S phase of DNA synthesis in normal and neoplastic ovarian cells (Massague, 1992). In addition to the cell cycle inhibition, TGF-β induced apoptosis in epithelial ovarian cancer but not in normal OSE, suggesting that neoplastic cells are more susceptible to apoptosis than their normal counterparts (Havrilesky *et al.*, 1995, Lafon *et al.*, 1996). The present study indicates that both exogenous activin and TGF-β induced apoptosis in neoplastic OSE cells that were growth-inhibited *in vitro*. It is hypothesized that growth inhibition by activin or TGF-β may be derived from induction of apoptosis in this model, suggesting that apoptosis may be also one of important phenomena in growth-inhibited ovarian cancer cells.

The bcl-2 gene family has been widely reviewed as regulators of cell death (reviewed in Chao and Korsmeyer, 1998; Minn *et al.*, 1998). Among pro- and anti-apoptotic genes in the bcl-2 family, bax and bcl-2 genes are dominant regulators of apoptosis. The ratio of bcl-2 to bax is important in determining susceptibility to apoptosis (Chao and Korsmeyer, 1998). It has been shown that steroid hormones and growth factors may regulate pro- or anti-apoptotic genes in ovarian and breast cancer cells (Lafon *et al.*, 1996; Lapointe *et al.*, 1999; Wang and Phang, 1995). The present study has demonstrated that bax and bcl-2 mRNAs are expressed in IOSE cell lines. No difference was observed in the expression level of bax mRNA between IOSE-29 and IOSE-29EC cells. In contrast, the expression level of bcl-2 mRNA is higher in IOSE-29EC cells than IOSE-29 cells, suggesting that IOSE-29EC cells may be more resistant to apoptosis. Relatively high expression levels of bcl-2 in IOSE-29EC cells suggest that this cell line is more resistant to serum deprivation than IOSE-29 cells (data not shown). To examine the exact mechanism by which activin and TGF-β regulates apoptosis in neoplastic OSE cells, the regulation of pro-apoptotic bax and anti-apoptotic bcl-2 protein was investigated following treatments of activin and TGF-β, respectively. Treatments of TGF-β (1 and 10 ng/ml) resulted in a significant decrease in bcl-2 protein (up to 50%), whereas no difference was observed in bax protein level. These findings are in agreement with a previous report where TGF-β1 down-regulated the endogenous expression of anti-apoptotic bcl-2 gene (Lafon *et al.*, 1996). Thus, down-regulated bcl-2 may elicit apoptosis in IOSE-29EC cells, suggesting that anti-apoptotic bcl-2 appears to be a dominant regulator of apoptosis in these cells. However, no difference was observed in bax and bcl-2 protein expression following treatments of increasing doses of activin. It has been reported that the expression of the pro-apoptotic bax was unchanged after activin treatment in B-cell lymphoma (Koseki *et al.*, 1995), however, over-expression of bcl-2

suppressed activin-induced apoptosis. Thus, different pro- and/or anti-apoptotic genes or possibly another apoptotic pathway may be related with activin-induced apoptosis in our culture system (Chao and Korsmeyer, 1998; Koseki *et al.*, 1998).

In conclusion, the present study indicates that both activin and TGF-β induced growth inhibition and apoptosis in experimentally produced early neoplastic (IOSE-29) and tumorigenic (IOSE-29EC) OSE cells. Furthermore, anti-apoptotic bcl-2 protein was down-regulated by TGF-β, whereas no difference was observed in bax protein by activin or TGF-β and in bcl-2 protein by activin. These results suggest that activin and TGF-β may play a role in growth inhibition and induction of apoptosis in early neoplastic and tumorigenic transformation stages of ovarian cancer.

3. *Experiment C*

The present studies demonstrated that both mRNAs and proteins of ERα and ERβ are expressed in IOSE cell lines by semi-quantitative RT-PCR and immunoblot analysis. No difference in the expression levels of ERs was observed among early neoplastic OSE, tumorigenic OSE, late neoplastic OSE and OVCAR-3 cells. In the previous studies, it has been demonstrated that human normal OSE cells express both mRNAs and proteins of ERα and ERβ (Brandenberger *et al.*, 1998; Kang *et al.*, 2001; Lau *et al.*, 1999). In addition, the expression levels of ERα were enhanced when compared to those in normal ovaries, whereas ERβ levels were significantly decreased in ovarian tumors, suggesting that ERα and ERβ mRNAs are differentially expressed in normal and neoplastic OSE cells (Brandenberger *et al.*, 1998; Pujol *et al.*, 1998). These results suggest that overexpression of ERα relative to ERβ mRNA may be a marker of ovarian tumorigenesis. Consistent with mRNA levels, ER protein is also highly

expressed in ovarian carcinomas when compared to normal or benign ovarian tumors (Tropila *et al.*, 1986). The open reading frame predicted from the ERβ cDNAs encodes a protein of molecular weight of approximately 54 kDa, which contrasts with the size of ERα (approximately 67 kDa) detected by Western blotting (Green *et al.*, 1986; Kuiper *et al.*, 1996). ERα and ERβ can homodimerize (α/α or β/β) or heterodimerize (α/β) upon binding to the ERE (Cowley *et al.*, 1997). Thus, it is hypothesized that differential expression of ERα and ERβ in ovarian tumors may alter a responsiveness of estrogen or anti-estrogen treatment. Recently, a mutation involving a 32-bp deletion in exon 1 of ERα transcripts was detected in SKOV-3 cell line, which is not responsive to estrogen treatment even though this cell line is ER-positive (Lau *et al.*, 1999).

In addition to its well-documented role in reproductive organs, it has been suggested that estrogen, especially 17β-estradiol (E2), may be associated with ovarian tumorigenesis. Treatments with exogenous estrogen resulted in a growth stimulation of several ER-positive ovarian carcinoma cell lines *in vitro* (Chien *et al.*, 1994; Galtier-Dercure *et al.*, 1992; Langon *et al.*, 1994). Some cultures of human epithelial ovarian cancer cells have been demonstrated to produce E2 and progesterone (Wimalasena *et al.*, 1991). The present studies demonstrated that exogenous E2 (10^{-8} - 10^{-6} M) resulted in an increased thymidine incorporation and DNA content in IOSE-29EC cells but not in IOSE-29 cells. The effect of E2 was attenuated by the estrogen antagonist tamoxifen (10^{-6} M), suggesting that the effect of E2 is mediated through specific receptors. As there was no stimulatory effect on thymidine incorporation prior to day 6, and since the ratio of thymidine incorporation / DNA content per culture did not change, E2 does not stimulate proliferation. The growth of ER-positive ovarian tumors that are responsive to E2 is also attenuated by antiestrogen, such as tamoxifen and the pure antiestrogen ICI 164,384

(Clinton and Hua, 1997; Langdon *et al.*, 1990). It is not yet known which ERs (α, β or both) are blocked by tamoxifen treatment.

In the present study, E2 does not appear to be mitogenic for IOSE-29EC cells even though E2 resulted in a significant increase in thymidine incorporation after 6-day treatment, because the increase in thymidine incorporation was paralleled by an increase in DNA content per culture. In addition, no difference in proliferative index was obtained after E2 treatment for 1 or 2 days (data not shown). These observations suggest that the increase in thymidine incorporation and DNA content may be due to reduced apoptosis. This increase in thymidine incorporation could reflect the stimulation of proliferation by E2 (i.e. an increase in the proportion of dividing cells per total cell number) or it could be the result of an unchanged rate of proliferation in cell populations that had increased in size because apoptosis was inhibited. The observation that a significant increase in thymidine incorporation was only observed on day 6 of E2 treatment supports the latter possibility. To define the basis for the increase in thymidine incorporation more definitely, total DNA determination was carried out on the cell populations. These determinations showed an increase in DNA content that paralleled the changes in thymidine incorporation, i.e. the ratio of thymidine incorporation over total cell number did not change. Therefore, it appears that the increase in cell number on day 6 was the result of suppression of apoptosis rather than enhanced proliferation. Further, in the work presented here, treatment with tamoxifen (10^{-6} M) only resulted in a growth-inhibitory effect in both IOSE-29 and IOSE-29EC cells, regardless of E2 treatment. Clinically relevant concentrations of tamoxifen (10^{-7}–10^{-5} M) have been shown to inhibit the growth of the ER-negative ovarian cancer cell line, A2780, and to induce apoptosis (Ercoli *et al.*, 1998). This estrogen-independent role of tamoxifen in ER-negative ovarian and breast cancer cells have been well documented (Ercoli *et al.*, 1998; Kang *et*

al., 1996; Markman *et al.*, 1996), suggesting that tamoxifen (10^{-6} M) may have dual functions, antagonizing the effects of estrogen by blocking ER and inhibition of growth through an estrogen-independent manner, as demonstrated in this experiment. In addition, tamoxifen may have estrogenic and antiestrogenic effects even in the same tissue. It has been demonstrated tamoxifen induces progesterone receptors in breast tissue (agonistic effect), but inhibits the growth of breast cancer (antagonistic effect). This cell-specific effect of tamoxifen in the same tissue can be explained by the tripartite theory (ligand, receptor, and effector) based on the cell- and promoter specific action of steroid hormones (Katzenellenbogen *et al.*, 1996), indicating that the biocharacter of ligand (i.e., agonist-antagonist balance) is determined principally through this receptor-effector coupling. This molecular explanation of the agonistic and antagonistic effects of the ligand-binded estrogen receptor is further supported by other groups (Brzozowski et al., 1997; Parker, 1998). In the present study, our results confirm those of others (Karlan *et al.*, 1995), which indicated that E2 does not affect the growth of normal OSE. The role of ER's in OSE and IOSE-29 remains to be further elucidated, but our results suggest that the introduction of E-cadherin resulted in an altered ER-elicited effect and responsiveness to E2 or tamoxifen resembling that of ovarian cancer lines.

Dysregulation of proliferation and/or cell death plays a critical role in tumorigenesis. The present study demonstrates that tamoxifen (10^{-6} M) can induce apoptosis in IOSE-29EC cells, whereas E2 can attenuate the effect of tamoxifen on these cells. Only IOSE-29EC cells were used for further studies of apoptosis because this cell line expressed both ERs and responded to E2/tamoxifen treatments. Co-treatment with E2 (10^{-8} to 10^{-6} M) plus tamoxifen attenuated tamoxifen-induced apoptosis in a dose-dependent manner. Among the pro- and anti-apoptotic genes in the bcl-2 family, bax and bcl-2 genes are dominant regulators for apoptosis. The ratio of

bcl-2 to bax is important in determining susceptibility to apoptosis (Chao and Korsmeyer, 1998). As an alternative pathway for apoptosis, Fas-Fas ligand system (CD95/CD95 ligand) mediates apoptosis in several tissues and tumors. Fas ligand is a type II integral membrane protein that bears homology to TNF-α, and both can be released from the surface as soluble cytokines (Herr et al., 1999). In addition, TNF-related apoptosis-inducing ligand (TRAIL) is another member of the TNF family of cytokines and mediates rapid apoptosis in transformed cell lines of various origin (Nagata, 1997). Among members of the TNF receptor superfamily, the intracellular "death domain" is highly conserved. The Fas associating protein with death domain (FADD), a major adaptor molecule, binds either directly or indirectly to the death domains of Fas, TNF and TRAIL receptors to tranduce the apoptosis signal to caspases (Nagata, 1997).

The present study has demonstrated that bax and bcl-2 are expressed at both mRNA and protein levels in neoplastic OSE cells. No difference was observed in the expression level of bax mRNA between IOSE-29 and IOSE-29EC cells. Interestingly, the expression level of bcl-2 mRNA and protein is higher in IOSE-29EC cells than IOSE-29 cells, suggesting that IOSE-29EC cells may be more resistant to apoptosis. In addition, treatments with E2 resulted in a significant increase in bcl-2 mRNA (up to 2-fold), whereas the effect of E2 was attenuated with tamoxifen treatment, suggesting that the up-regulation of bcl-2 mRNA by E2 is mediated through specific estrogen receptors. These findings are in agreement with a previous report where estrogen up-regulated anti-apoptotic bcl-2 gene, while bax levels remain unaffected by E2 in breast cancer cells (Leung and Wang, 1999; Perillo *et al.*, 2000; Teixeira *et al.*, 1995; Wang and Phang, 1995). The up-regulation of bcl-2 by E2 in this series of experiments indirectly suggests that E2 affects the survival of IOSE-29EC cells through bcl-2 which is known to be a dominant regulator of apoptosis in other tissues (Leung and Wang, 1999; Perillo *et al.*, 2000;

Teixeira *et al.*, 1995; Wang and Phang, 1995). It has been shown that estrogen down-regulated pro-apoptotic bak and anti-apoptotic bcl-X_L mRNA and protein in a dose-dependent manner, suggesting different members of bcl-2 family may be regulated *via* different pathways by estrogen (Leung and Wang, 1999). In parallel with the mRNA level, E2 induced a significant up-regulation of bcl-2 protein level (up to 1.7-fold), whereas no difference was observed in bax mRNA level. This induction of bcl-2 protein by E2 was attenuated with tamoxifen treatment (10^{-6} M). Thus, the mechanism by which estrogen regulates the apoptotic pathway may be related to up-regulation of the bcl-2 gene. Recently, it has been demonstrated that the bcl-2 major promoter does not contain cis-acting elements, which are directly involved in transcriptional control by E2, and that E2 induces bcl-2 expression via two estrogen-responsive elements located within its coding region (Perillo *et al.*, 2000).

In conclusion, the present study indicates that early neoplastic (IOSE-29), tumorigenic (IOSE-29EC) and late neoplastic (IOSE-29EC/T4 and T5) OSE cell lines, which were generated from normal OSE, express both ERα and ERβ at the mRNA and protein levels. E2 has been demonstrated to prevent tamoxifen induced-apoptosis through ERs. The mechanism of action of E2 may be associated with up-regulation of bcl-2 gene at the mRNA and protein levels. These results suggest that estrogen may play a role in the prevention of apoptosis in tumorigenic OSE cells for ovarian tumorigenesis.

4. Experiment D

In addition to its well-documented role in ovarian physiology, FSH, one of the pituitary glycoprotein hormones, has been suggested to play a role in ovarian cancer development (Konishi *et al.*, 1999; Zheng *et al.*, 2000). An increased occurrence of ovarian cancer with

exposure to high levels of gonadotropins during postmenopause or infertility therapy has been suggested by epidemiological studies (Risch, 1998; Shushan *et al.*, 1996; Whittemore *et al.*, 1992). Little information is available regarding the expression of FSH-R and exact role of FSH in normal and neoplastic OSE cells. FSH-R, a G-protein coupled receptor, is expressed in normal OSE (Zheng *et al.*, 1996), ovarian inclusions and epithelial tumors (Zheng *et al.*, 2000). In addition, treatment with FSH resulted in growth-stimulation of rabbit OSE (Osterholzer *et al.*, 1985) and ovarian cancer cells (Wimalasena *et al.*, 1992; Zheng *et al.*, 2000) in a dose- and time-dependent manner *in vitro*. In agreement with these reports, the present study demonstrated that FSH-R mRNA was expressed and that treatment of FSH induced a growth-stimulation in both normal and neoplastic OSE cells. It has been shown that FSH-R expression was decreased with increasing tumor grade in epithelial inclusions, cystadenomas, borderline tumors and carcinomas, suggesting that constitutive expression of FSH-R may represent a cellular differentiation marker for epithelial ovarian tumors (Zheng *et al.*, 2000). However, in the present study, it appears that no difference was observed in the expression level of FSH-R in normal, early neoplastic (IOSE-29), tumorigenic (IOSE-29EC) and late tumorigenic OSE (IOSE-29EC/T4 and /T5) cells by RT-PCR and Southern blot analysis. A recent report demonstrated that elevated level of gonadotropins stimulated growth of ovarian carcinoma by induction of tumor angiogenesis, and the FSH effect was connected with the expression of vascular endothelial growth factor (VEGF), which is an angiogenic factor presumably involved in ovarian tumorigenesis (Schiffenbauer *et al.*, 1997), implying that gonadotropins may facilitate the growth of existing microtumors by enhancing blood supply.

Protein phosphorylation is a critical regulatory response to cellular stimulation and differentiation. MAPK cascade is known to regulate acute cellular responses and to control

transcriptional events through phosphorylation of target enzymes and transcriptional factors (Biesen *et al.*, 1996; Cobb and Goldsmith, 1995; Davis, 1994). Activation of ERK is induced by phosphorylation of both threonine and tyrosine residues of the enzymes as a result of stimulation of Ras, ERK kinase kinase, MEK kinase and MEK (Cobb and Goldsmith, 1995; Davis, 1994; Seger and Krebs, 1995). The MAPKs have been shown to mediate a diverse range of regulatory molecules such as FSH (Das *et al.*, 1996), prostaglandin F2α (Chen *et al.*, 1998), TGF-α (Sasanami *et al.*, 1999), or EGF (McClellan *et al.*, 1999) in the ovarian cells. In addition, treatment with GnRH analog (GnRHa) resulted in a sustained (24 h) activation of ERK, while PD98059, which binds MEK, blocked GnRHa-induced growth inhibition as well as hypo-phosphorylation of pRB in CaOV-3 cells (Kimura *et al.*, 1999). As the mechanism of FSH action in ovarian tumors is not clear, we investigated the possible regulatory action of FSH on MAPK activation and its role in neoplastic OSE cells. In the present study, FSH stimulated MAPK activation in both IOSE-29 and IOSE-29EC cells, whereas the stimulatory effect of FSH was reversed completely by pretreatment with PD98059, an ERK inhibitor, suggesting that the growth stimulatory effect of FSH may be mediated by the MAPK pathways in neoplastic OSE cells. In previous reports, exogenous EGF activated ERK1/2, increased and sustained levels of *c-jun* mRNA, but had no effect on JNK1 activation in IOSE-29 cells (McClellan *et al.*, 1999). Similarly, EGF has been demonstrated to induce activation of ERK and cellular proliferation was partially inhibited by PD98059 in a prostate cancer cell line (Price *et al.*, 1999). Additionally, it has been shown that EGF-induced cell proliferation, MMP-9 induction and invasion through reconstituted basement membrane were significantly reduced when breast epithelial cells were exposed to MEK inhibitor (PD 98059) or MAPK inhibitors (Apigenin or MAPK antisense phosphorothioate oligodeoxynucleotides). These results suggest that interference with MAPK

activity may affect the growth and invasiveness of tumors in which the signaling cascade is activated (Reddy *et al.*, 1999). Whether interference of the MAPK cascade with ERK or MAPK inhibitors may induce a growth-inhibition in normal and neoplastic OSE cells warrants future investigation.

In a time-dependent study, treatment with FSH induced a significant increase in MAPK activation at 5-10 min in IOSE-29 cells. The activated MAPK declined to control level after 20 min in these cells. In contrast, treatment with FSH significantly induced MAPK activation after 5 min and the activity was sustained for 60 min in IOSE-29EC cells. It appears that cellular responses to MAPK may be influenced by the duration of its activation. Sustained activation of MAPK is associated with cellular differentiation by nerve growth factor (NGF) in PC12 cells, whereas transient activation of MAPK by epidermal growth factor (EGF) leads to cellular proliferation (Heasley and Johnson, 1992; Nguyen *et al.*, 1993). Thus, a rapid activation of MAPK by FSH in IOSE-29 and IOSE-29EC cells is related with a growth-stimulation in the present study. However, the cause of sustained response following FSH treatment in IOSE-29EC cells has yet to be elucidated. EGF stimulated an early rise in ERK activity at 4 min followed by a rapid decline in normal breast epithelium, whereas a sustained (1 h) elevation of ERK activity was observed in the tumor cells (Xing and Imagawa, 1999), suggesting the time course of ERK activity may be different between normal and neoplastic cells. In addition, PD98059 inhibited EGF-stimulated proliferation and ERK activity in a parallel, dose-dependent manner, indicating that ERK activation is at least permissive for the proliferative response to EGF (Xing and Imagawa, 1999).

FSH-R belongs to a superfamily of G-protein coupled receptors, which interact with intracellular signaling system *via* 7-transmembrane domains (Simoni *et al.*, 1997). Transient

increase of *c-fos, c-myc* expression and MAPK activation were demonstrated in granulosa cell cultures in response to FSH (Cameron *et al.*, 1996; Das *et al.*, 1996; Pennybacker and Herman, 1991). The actions were mediated by either cAMP-dependent or independent pathway. The MAPK pathway has been shown to mediate the cAMP-independent FSH induced growth promotion (Babu *et al.*, 2000). In the present study, staurosporin, a PKC inhibitor, was employed to investigate whether FSH-activated MAPK is mediated by cAMP independent and PKC dependent pathway (Watson *et al.*, 1988; Melner, 1996). The FSH-induced activation of MAPK was completely blocked by pretreatment with staurosporin (1 μM) for 30 min in both cell lines, suggesting that FSH acts *via* a PKC pathway in neoplastic OSE cells. As demonstrated in this study, FSH did not stimulate basal cAMP level, suggesting that the PKA pathway is not involved in the FSH-induced MAPK activation in neoplastic OSE cells. This is in contrast to human granulosa-luteal cells where FSH stimulates cAMP accumulation and MAPK activation occurs via a PKA-dependent pathway. In other ovarian cancer cell lines examined, it appears that FSH induced a significant increase in MAPK activation in OVCAR-3 cells, not in SKOV-3 cells. Pre-treatment with PD98059 or staurosporin resulted in a decrease in FSH-induced MAPK activation in OVCAR-3 cells, whereas pre-treatment with PD98059 or staurosporin did not affect on FSH-induced MAPK activity in SKOV-3 cells.

Several studies have shown that MAPKs phosphorylate ternary complex factor (TCF) proteins such as Elk-1 and SAP-1 (Gille *et al.*, 1995; Janknecht *et al.*, 1993, Treisman, 1994). The activated TCF protein regulates the expression of c-fos and other co-regulated genes through their actions on the serum response element. Therefore, the ability of FSH to activate a downstream effector of the MAPK pathway was examined using immunoprecipitation. The present study demonstrated that treatment with FSH resulted in substantial phospohorylation of

Elk-1 fusion protein *in vitro*. These results confirmed that FSH action is mediated by the MAPK pathway, as treatment with PD98059 completely reversed the effect of FSH on Elk-1 phosphorylation. Taken together, these results suggest that FSH-stimulated MAPK activation resulted in phosphorylation of Elk-1, the Ets family of transcription factors, which possibly mediates cellular functions in response to FSH in neoplastic OSE cells.

In conclusion, we demonstrated that FSH-R was expressed and FSH induced growth-stimulation in both normal and neoplastic OSE cells. In addition, FSH activated the MAPK cascade presumably through a PKC-dependent pathway. Activated MAPK phosphorylated Elk-1 in neoplastic OSE cells. These results suggest that MAPK cascade may be involved in cellular function such as growth stimulation in response to FSH in OSE cells.

5. *Experiment E*

Epithelial ovarian tumors are the most common cause of death from gynecological malignancies and appear to arise from OSE, which is a simple squamous-to-cuboidal mesothelium covering the ovary based on histopathological observations (Auersperg *et al.*, 1995). The exact mechanism of ovarian tumorigenesis has not been elucidated, but repeated ovulation and process of healing ruptured OSE have been suggested to contribute to neoplastic transformation of OSE (Godwin *et al.*, 1993). Considering the fundamental role of OSE cells in ovarian tumorigenesis, the growth regulation of normal and neoplastic OSE cells by intraovarian regulators may play an important role in ovarian cancer development. Recent cloning of a second form of GnRH has been demonstrated in the brain (Lescheid *et al.*, 1997; Urbanski *et al.*, 1999) and expressed at significantly higher levels outside the brain (White *et al.*, 1998). Therefore, it is tempting to investigate the role of GnRH-II in normal and neoplastic OSE cells, which will

contribute to a better understanding of normal ovarian physiology and the role of OSE in ovarian tumorigenesis.

In the present study, we demonstrated for the first time that GnRH-II is expressed in normal and neoplastic OSE cells, suggesting that GnRH-II exerts its actions in an autocrine/paracrine manner. Sequence analysis indicated that GnRH-II in these cells have a nucleotide sequence identical to that of other tissues (White *et al.*, 1998). The presence of GnRH-II in normal OSE as wells as neoplastic OSE indicates that the local regulatory system based on GnRH-II in normal OSE is a normal component of the cells. In a previous report, it has been demonstrated that GnRH-II is expressed and down-regulates FSH/LH receptors in human granulose luteal cell (Kang *et al.*, 2001), suggesting that GnRH-II may have biological effects similar to those of GnRH-I. The present study demonstrated that GnRH-R is expressed in these cell lines, suggesting that the actions of GnRH-I and –II are a receptor-mediated event. GnRH-II binds GnRH-R up to 100 times more effectively than GnRH-I, suggesting GnRH-II may act through GnRH-R outside the brain (King and Millar, 1991). One interesting finding in the present study was the demonstration that treatments (10^{-9} – 10^{-7}) with GnRH-I and –II resulted in a growth-inhibitory effect in IOSE-29 and IOSE-29EC cells. It has been demonstrated previously that GnRH has antiproliferative effects and regulates its own receptor mRNA, suggesting that GnRH-I may act as an autocrine regulator in normal OSE cells (Kang *et al.*, 2000). In addition, GnRH analogs have been proved to be efficient in treating GnRH-R bearing tumors, including carcinomas of the ovary (Emons *et al.*, 1993a; Yano *et al.*, 1994). These results indicate that GnRH-II may play a role in growth-inhibition similar to GnRH-I in normal and neoplastic OSE cells. The mechanism of growth-inhibitory effect of GnRH-II remains to be elucidated. It has been demonstrated GnRH analogs reduce cell proliferation by increasing the portion of cells in

the resting phase, G_0-G_1 (Thomson *et al*, 1991) and by inducing cell death or apoptosis (Motomura, 1998; Sridaran *et al.*, 1998). Treatment of ovarian cancer cells with GnRH analogues may induce apoptosis mediated by the Fas ligand-Fas system, which has been shown to trigger apoptosis in a variety of cell types (Nagata and Golstein, 1995). In this study, treatment with GnRH-II (10^{-7} M) resulted in an induction of apoptosis in IOSE-29 and IOSE-29EC cells by DNA fragmentation assay. In addition, co-treatment with antide (10^{-7} M) and GnRH-II completely blocked the effect of GnRH-II on the induction of DNA fragmentation, suggesting that GnRH-II may trigger a cellular signaling pathway *via* GnRH-R. However, no significant change in bax and bcl-2 proteins was observed following GnRH-II (10^{-7} M) treatment, suggesting that another pathway may be involved in terms of the induction of apoptosis by GnRH-II in neoplastic OSE cells. More studies of the effect of GnRH-II on apoptosis in normal and neoplastic OSE cells need to be extended.

In conclusion, we demonstrated that GnRH-II is expressed in normal and neoplastic OSE cells. In addition GnRH-II induces a growth-inhibition and apoptosis in neoplastic OSE cells. These results suggest that GnRH-II may be an integral regulator in normal OSE physiology and play a role in a growth-inhibition in neoplastic OSE cells.

V. SUMMARY AND FUTURE STUDIES

1. Summary

The exact mechanism of ovarian tumorigenesis is not well known even though this disease is the most frequent cause of cancer death in gynecological malignancies. Repeated ovulation contributes to neoplastic transformation of OSE, indicating that the process of healing ruptured OSE may contribute to the disease. Therefore, it has been hypothesized that endocrine and autocrine factors may influence the occurrence of ovarian tumors in women. Recently, non-tumorigenic and tumorigenic immortalized OSE (IOSE) cells were generated from normal OSE directly by transfection with simian virus 40 (SV40)-large T antigen (IOSE-29) and subsequent E-cadherin (IOSE-29EC). These IOSE-29EC cells were found to be anchorage independent and formed transplantable, invasive subcutaneous and intraperitoneal adenocarcinomas in SCID mice. Thus, two additional cell lines, designated IOSE-29EC/T4 and IOSE-29EC/T5 were established from tumors that arose in IOSE-29EC-inoculated SCID mice. This study was performed to investigate the effects of activin, TGF-β, estradiol (E2), Follicle-stimulating hormone (FSH) and gonadotropin-releasing hormone-II (GnRH-II) in the growth-stimulation or -inhibition and regulation of apoptosis in normal and neoplastic OSE cells using IOSE cell lines.

This study indicates that α and βA subunits were highly expressed in normal OSE compared to OVCAR-3 cells, whereas βB subunit was highly expressed in OVCAR-3 cells, compared to normal OSE cells. In addition, activin receptor IIB mRNA levels were significantly higher in OVCAR-3 compared to normal OSE cells. At concentrations of 1, 10 and 100 ng/ml, rh-activin A stimulated the growth of OVCAR-3 cells, but not of normal OSE. Treatment with follistatin, binding protein of activin, attenuates the stimulatory effect of activin. Treatments with activin

increased the α and βA subunit mRNA levels in a dose- and time-dependent manner. But, no difference was observed in levels of βB subunit, or in activin receptor type IIA and IIB mRNAs following activin treatments in OVCAR-3 cells. These results suggest that different levels of activin/inhibin and activin receptor isoforms are expressed in normal and neoplastic OSE cells. In addition, the altered expression of the activin/inhibin subunits, as well as the cell proliferative effect of activin observed in OVCAR-3 but not in normal OSE cells, indicate that activin may act as an autocrine regulator of neoplastic OSE progression.

In IOSE cell lines, activin/inhibin subunits and activin receptors were expressed at both the mRNA and protein levels in these cells. Treatments with activin (1-100 ng/ml) resulted in a significant decrease in cellular proliferation in both IOSE-29 and IOSE-29EC cells, though we have shown that it had no effect in normal OSE. This inhibitory effect was attenuated by co-treatment with follistatin. In addition, treatment with TGF-β (0.1-10 ng/ml) also significantly decreased the proliferation of normal, IOSE-29 and IOSE-29EC in a dose-dependent manner. Treatments with both activin and TGF-β resulted in an increase in DNA fragmentation in IOSE-29EC cells in a dose-dependent manner. This apoptotic effect of activin was attenuated with co-treatment by follistatin. Furthermore, treatment with TGF-β (1 and 10 ng/ml) resulted in a significant decrease in bcl-2 protein (up to 50%) in IOSE-29EC, whereas no difference was observed in bax protein levels. Therefore, down-regulation of bcl-2 by TGF-β may eventually induce apoptosis in IOSE-29EC cells. No difference was observed in bax and bcl-2 protein expression following treatment with activin. In conclusion, the present study indicates that activin and TGF-β inhibited growth and induced apoptosis in early neoplastic (IOSE-29) and tumorigenic OSE (IOSE-29EC) cells. In addition, anti-apoptotic bcl-2 protein was down-regulated by TGF-β, whereas no difference was observed in bax protein by activin or TGF-β

treatment and in bcl-2 protein by activin. These results suggest that activin and TGF-β may play a role in growth inhibition and induction of apoptosis in early neoplastic and tumorigenic stage of ovarian cancer.

Both mRNAs and proteins of estrogen receptor (ER) α and β were expressed in IOSE cell lines. No difference was observed in normal OSE and IOSE-29 cells, whereas treatment with 17β-estradiol (E2, 10^{-8} - 10^{-6} M) resulted in an increased thymidine incorporation and DNA content per culture in IOSE-29EC cells. This effect of E2 was attenuated with tamoxifen treatment (10^{-6} M), an estrogen antagonist, suggesting that the effect of E2 is mediated through specific ERs. There was no stimulatory effect on thymidine incorporation prior to day 6, but after 6 days of E2 treatment, thymidine incorporation was significantly increased. Since the ratio of thymidine incorporation / DNA content per culture did not change, this E2 effect does not appear to indicate stimulation of proliferation, but, rather, inhibition of apoptosis. Treatment with tamoxifen (10^{-6} M) induced apoptosis up to 3-fold in IOSE-29EC cells, whereas co-treatment with E2 (10^{-8} to 10^{-6} M) plus tamoxifen attenuated tamoxifen-induced apoptosis in a dose-dependent manner. Both mRNA and protein levels of pro-apoptotic bax and anti-apoptotic bcl-2 were expressed in IOSE cell lines. Treatments with E2 resulted in a significant increase in bcl-2 mRNA and protein levels (2 and 1.7-fold respectively), whereas no difference was observed in bax mRNA level. Thus, E2 may enhance survival of IOSE-29EC by up-regulating bcl-2. Anti-apoptotic bcl-2 may be a dominant regulator in the apoptotic pathway in these cells. In conclusion, the present study indicates that early neoplastic (IOSE-29), tumorigenic (IOSE-29EC) and late neoplastic (IOSE-29EC/T4 and T5) OSE cells expressed both ERα and ERβ at the mRNA and protein levels. In addition, E2 prevented tamoxifen induced-apoptosis through ERs. The mechanism of E2 action may be associated with up-regulation of bcl-2 gene at mRNA

and protein levels. These results suggest that estrogen may play a role in ovarian tumorigenesis by preventing apoptosis in tumorigenic OSE cells.

In the present study, FSH-R mRNA was expressed in normal and neoplastic OSE cells. FSH exerted a growth-stimulatory effect in both normal and neoplastic OSE cells. Treatment with FSH activated MAPK in immortalized OSE cell lines (IOSE-29 and IOSE-29EC), whereas the stimulatory effect by FSH was completely abolished in the presence of PD98059, a MEK inhibitor, suggesting that the growth stimulatory effect of FSH may be mediated by MAPK activation in neoplastic OSE cells. In a time-dependent study, FSH significantly increased MAPK activity at 5-10 min in IOSE-29 cells. The activated MAPK declined to control levels after 20 min in these cells. In contrast, treatment with FSH significantly activated P-MAPK after 5 min and the activation was sustained for 60 min in IOSE-29EC cells. FSH-stimulated MAPK activity was completely blocked by pretreatment with staurosporin (1 μM), a protein kinase C (PKC) inhibitor, for 30 min in both cell lines. Treatment with FSH did not affect basal intracellular cAMP levels in IOSE-29 and IOSE-29EC cells, suggesting that the PKA pathway is not involved with FSH-induced MAPK activation in neoplastic OSE cells. A MAP kinase assay revealed that FSH resulted in substantial phosphorylation of Elk-1, confirming that FSH action is mediated by activation of MAPK. In conclusion, it has been demonstrated that FSH-R was expressed and FSH induced a growth-stimulatory effect in normal and neoplastic OSE cells. In addition, FSH stimulated the activation of MAPK cascade presumably through a PKC-dependent pathway. Activated MAPK phosphorylated Elk-1 in neoplastic OSE cells. These results suggest that the MAPK cascade may be involved in cellular function such as growth stimulation in response to FSH in neoplastic OSE cells.

GnRH-II mRNA was expressed in normal OSE, immortalized OSE (IOSE), ovarian tumors

from patients and ovarian cancer cell lines, suggesting that the actions of GnRH-II are exerted in an autocrine/paracrine manner in these cells. Treatment with increasing doses (10^{-9} – 10^{-7} M) of GnRH-I and –II resulted in a growth-inhibition of IOSE-29 and IOSE-29EC cells. In addition, treatment with GnRH-I (D-Ala-GnRH, 10^{-7} M) increased DNA fragmentation in both cell lines. Co-treatment of antide (10^{-7} M) and GnRH-I completely blocked the effect of GnRH-I on the induction of DNA fragmentation. Furthermore, treatment with GnRH-II (10^{-7} M) resulted in an increase in DNA fragmentation in IOSE-29 and IOSE-29EC cells. Similarly, co-treatment of antide (10^{-7} M) and GnRH-II completely blocked the effect of GnRH-II on the induction of DNA fragmentation. However, treatment with GnRH-I had no effect on both bax and bcl-2 proteins in both cell lines. In addition, no significant difference in bax and bcl-2 proteins was observed following GnRH-II treatments. These results suggest that GnRH-II may be an integral regulator similar to GnRH-I, in normal OSE physiology and play a role in the induction of growth-inhibition and apoptosis, *via* mechanisms other than bax and bcl-2, in neoplastic OSE cells.

Taken together, the above-mentioned studies have investigated the roles of activin, TGF-β, estradiol, FSH and GnRH-II as endocrine and autocrine regulators of proliferation and apoptosis in normal and neoplastic OSE cells. The proposed intracellular signaling cascades of activin, TGF-β, estradiol, FSH and GnRH-II have been suggested based on the conclusions in these studies (Fig. 59). These findings strongly suggest that these regulators may play a role in ovarian tumorigenesis in terms of the regulation of cell growth and/or cell death.

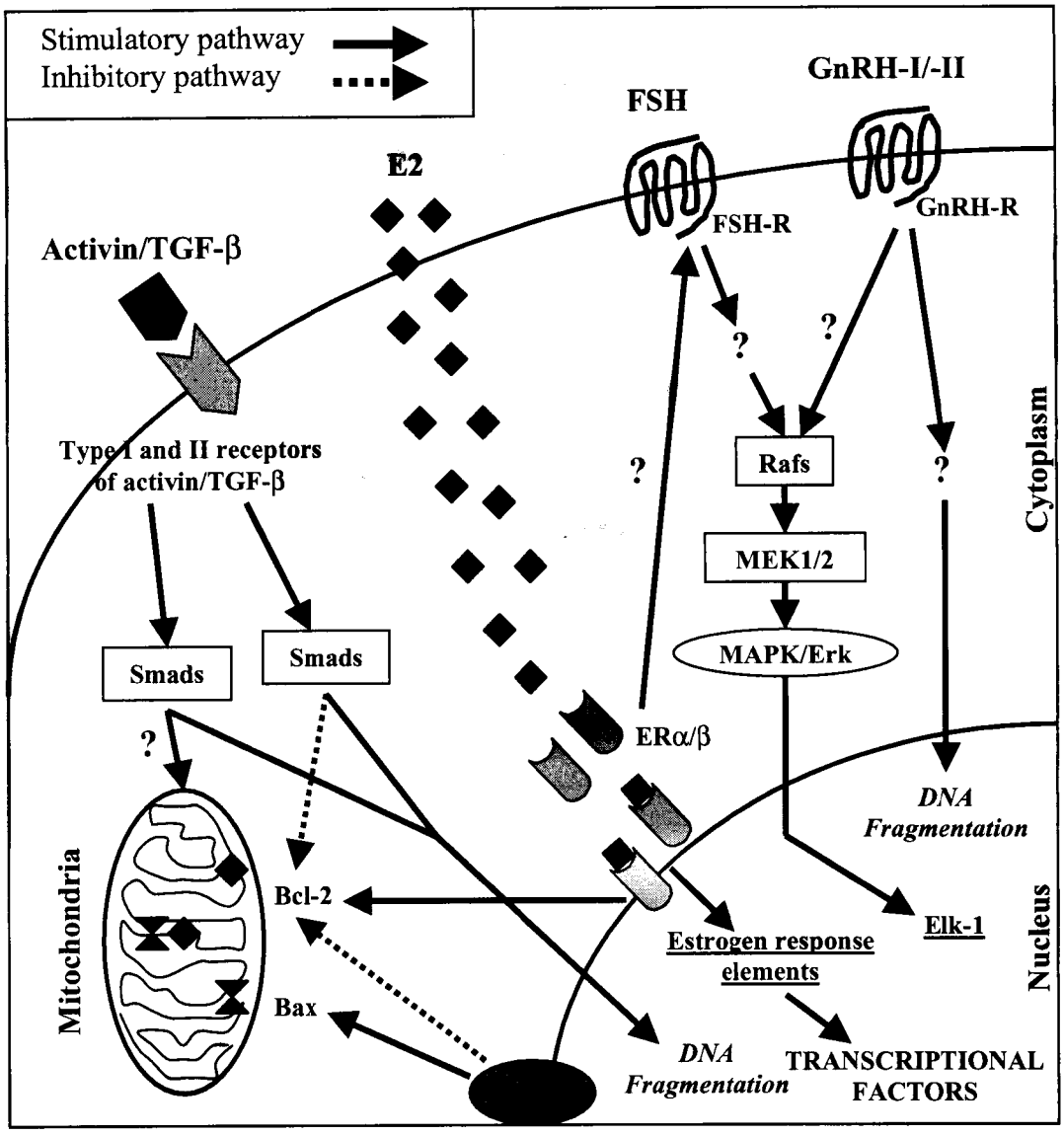


Figure 59. Proposed intracellular signaling cascades of activin, TGF-β, E2, FSH and GnRH-II in neoplastic OSE cells.

2. Future Studies

2.1. Intracellular signaling pathways of activin and TGF-β in normal and neoplastic OSE cells

Future studies are required to elucidate the intracellular signaling pathways involved in the growth-stimulatory or –inhibitory effects of activin and TGF-β in normal and neoplastic OSE cells. It has been demonstrated that OVCAR-3 cells express Smad-4 and Smad-2, proteins specific for the TGF-β superfamily signaling pathway (Ito *et al.*, 2000). Although no detectable change was induced in Smad-4 mRNA in OVCAR-3 cells, Smad-2 mRNA levels were increased following treatment with activin A (50 ng/ml) for 48 h. Therefore, it is tempting to identify whether these specific signaling pathways are activated following activin or TGF-β treatment in normal and immortalized OSE cells *in vitro*. Future experiments will provide a better understanding of the mechanisms involved in activin or TGF-β action in epithelial ovarian carcinoma.

2.2. Apoptotic pathways by activin and TGF-β in normal and neoplastic OSE cells

In the present study, treatment with activin and TGF-β induced growth inhibition and apoptosis in early neoplastic and tumorigenic OSE cells. The anti-apoptotic bcl-2 protein was down-regulated by TGF-β treatment, but not by activin treatment. No difference was observed in bax protein levels following treatment with either TGF-β or activin. Thus, different pro- and/or anti-apoptotic genes or possibly other apoptotic pathways may be related with activin-induced apoptosis in our culture system (Chao and Korsmeyer, 1998; Koseki *et al.*, 1998). The involvement of other pro- and anti-apoptotic genes such as bcl-X_L, bcl-W, bcl-X_S, bad, and bak

should be evaluated to explain the molecular mechanism of activin-induced apoptosis in neoplastic OSE cells.

2.3. Apoptotic pathways by Estradiol in tumorigenic OSE cells

In the present study, treatment with E2 prevented tamoxifen-induced apoptosis through ERs in tumorigenic OSE cells. The mechanism of action of E2 may be associated with up-regulation of bcl-2 gene at the mRNA and protein levels. Thus, future experiments are required to investigate the effect of E2 on downstream pathways of apoptosis such as caspase-3, caspase-9 and/or poly(ADP)-ribose polymerase in these cells. In addition, future experiments should clarify whether the bcl-2 major promoter contains cis-acting elements directly involved in transcriptional control by E2, and whether E2 induces bcl-2 expression *via* estrogen-responsive element(s) located within its coding region.

2.4. Involvement of PKC pathway in activating MAPK by FSH in neoplastic OSE cells

MAPK pathway has been shown to mediate the cAMP-independent FSH activation of growth promotion in granulosa tumors (Babu *et al.*, 2000). In the present study, treatment with FSH activated the MAPK (ERK-1/-2) cascade and phosphorylated Elk-1 in neoplastic OSE cells presumably through a PKC-dependent pathway. Involvement of PKC pathway in FSH-induced MAPK activation needs to be extensively examined using IOSE cell lines. In addition, activation of other transriptional factors including *c-fos and c-myc* by FSH treatment should be determined in these cells.

2.5. Apoptotic pathways by GnRH-II in normal and neoplastic OSE cells

In the present study, GnRH-II induces a growth-inhibitory and apoptotic effect in neoplastic OSE cells. However, no significant change in the levels of bax and bcl-2 proteins was observed following GnRH-II treatment, suggesting that another pathway may be involved in the induction of apoptosis by GnRH-II in neoplastic OSE cells. Thus, the involvement of Fas ligand-Fas system, demonstrated in GnRH-induced apoptosis in other tissues, should be evaluated to explain the molecular mechanism involved in GnRH-II induced apoptosis in neoplastic OSE cells.

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