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**EXPRESSION, REGULATION, AND FUNCTION OF THE KIT TYROSINE
KINASE RECEPTOR AND ITS LIGAND, STEM CELL FACTOR,
IN HUMAN EPITHELIAL OVARIAN CANCER**

by

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Thesis submitted to the School of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

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Dedication

I dedicate this Ph.D. thesis to my parents, Martin and Marilyn Tonary, whose

LOVE for me

SUPPORT of me

and

PRIDE in me

have nourished me my entire life. Thank you Mom and Dad!

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Abstract

The *c-KIT* protooncogene encodes a tyrosine kinase receptor, KIT, that is activated upon binding of its ligand, stem cell factor (SCF). In normal cells, the KIT:SCF interaction can lead to a myriad of biological responses, including proliferation, migration, maturation, differentiation, and survival. Numerous types of cancer cells also express *c-KIT*, and exposure of these cells to SCF also stimulates proliferation and survival. This Ph.D. project sought to determine the expression, regulation, and function of the KIT-SCF receptor-ligand system in human epithelial ovarian cancer, the most common type of ovarian cancer.

The expression of *c-KIT* and SCF in normal ovaries, in cultured ovarian surface epithelium (OSE), and in epithelial ovarian tumors was analyzed by northern and western blot analyses, reverse transcription-polymerase chain reaction, and immunohistochemistry. Normal OSE expressed SCF, but not *c-KIT*; however, epithelial invaginations and inclusion cysts often expressed KIT protein. Of 15 benign ovarian tumors and tumors of low malignant potential, 87% expressed *c-KIT*, and 92% of these co-expressed SCF, suggesting the possibility of autocrine growth regulation. Of 35 malignant ovarian cancers, 71% expressed *c-KIT* (92% co-expressed SCF), with a trend for decreased *c-KIT* expression in advanced stage disease. Of 34 patients with malignant tumors for whom follow-up information was available (median follow-up time of 24 months), 9 had tumors that did not express *c-KIT*, 8 (89%) of whom have died and the remaining 1 has recurrent disease. Of the 25 patients with tumors expressing *c-KIT*, 56% are still alive, eight of whom have no evidence of disease. Importantly, statistical analysis indicated that patients whose tumors did not express *c-KIT* had a significantly shorter ($p < 0.05$) disease-free survival time than patients who had KIT-expressing tumors.

Studies were carried out to identify intraovarian growth regulatory factors which may regulate *c-KIT* and SCF expression in ovarian cancer cells, and to determine whether activated KIT can affect the proliferation and survival of these cells. HEY cells, which co-expressed KIT and SCF, were treated with transforming growth factor (TGF)- α , TGF- β , and dibutyryl cyclic AMP (dbcAMP) and their cellular proliferation and expression of *c-KIT* and SCF were examined. Treatment of HEY cells with TGF- α stimulated proliferation and also decreased the expression of *c-KIT*. Proliferation and *c-KIT* expression by HEY cells was significantly decreased by treatment with TGF- β , while dbcAMP decreased HEY cell proliferation but increased *c-KIT* expression. The steady-state levels of HEY cell *c-KIT* mRNA were also regulated by the confluency of the cells in culture. Expression of SCF in HEY cells was not affected by the growth factors or dbcAMP, but was regulated by cell confluency. Additionally, the proliferation of both HEY and OVCA 429 cells (which also co-expressed KIT and SCF) was not influenced by exposure of the cells to an anti-KIT neutralizing antibody or SCF, likely due to an exclusively cytoplasmic localization of KIT protein in these cells.

A series of transfection studies were carried out to determine if enforced *c-kit* expression in human ovarian carcinoma cells could regulate cellular proliferation. Transient transfection of *c-kit* into HEY cells resulted in decreased proliferation. Similarly, stable transfection of *c-kit* into A2780-cp cells, which do not express endogenous *c-KIT*, also resulted in a decreased proliferative rate. In contrast to the ovarian cancer cells, increased proliferation was documented for NIH 3T3 fibroblast cells transiently transfected with *c-kit*.

Together, these results suggest that the positive prognostic value of *c-KIT* expression in ovarian tumors is related to its negative growth regulatory function in ovarian cancer cells.

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List of Abbreviations and Chemical Formulae

1 st	first
³ H	tritium
³² P	³² phosphorous
°C	degrees Celcius
γ	wavelength
α	alpha or anti
abs	absorbance
Ad	adenocarcinoma
α-MEM	alpha-Minimal Essential Medium
AML	acute myelogenous leukemia
ANOVA	analysis of variance
AR	androgen receptor
Asp	aspartic acid
ATP	adenosine triphosphate
bp	base pair
BSA	bovine serum albumin
cAMP	adenosine 3', 5'-cyclic monophosphate
CaCl ₂	calcium chloride
cDNA	copy DNA
c- <i>fms</i>	colony stimulating factor-1 receptor
cm ³	cubic centimetre
CO ₂	carbon dioxide
cpm	counts per minute
CSF-1	colony stimulating factor-1
DAB	diaminobenzidine
dATP	deoxyadenosine triphosphate
dbcAMP	dibutyl adenosine 3', 5'-cyclic monophosphate
dCTP	deoxycytidine triphosphate
ddH ₂ O	distilled, deionized water
dGTP	deoxyguanosine triphosphate
D-MEM/F-12	Dulbecco's Modified Eagle Medium:Nutrient Mixture F-12 (1:1)
D-PBS	Dulbecco's phosphate-buffered saline
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
dTTP	deoxythymidine triphosphate
DTT	dithiothreitol

E	estrogen
E-cadherin	epithelial cadherin
EDTA	ethylenediaminetetraacidic acid
EGF	epidermal growth factor
EGF-R	epidermal growth factor receptor
ER	estrogen receptor
FBS	fetal bovine serum
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FSH	follicle-stimulating hormone
FSH-R	follicle-stimulating hormone receptor
g	gram
G	gauge
GAPDH	glyceraldehyde phosphate dehydrogenase
G-CSF	granulocyte-colony stimulating factor
GDF-9	growth differentiation factor-9
g/l	gram per litre
GM-CSF	granulocyte macrophage-colony stimulating factor
GnRH	gonadotropin-releasing hormone
h	hour
H ₂ O ₂	hydrogen peroxide
hCG	human chorionic gonadotropin
HCl	hydrochloric acid
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HRP	horseradish peroxidase
IgG	immunoglobulin
IMDM	Iscoe's Modified Eagle Medium
IL	interleukin
kb	kilobase
kD	kilodalton
KCl	potassium chloride
KH ₂ PO ₄	potassium dihydrogen orthophosphate
l	litre
LH	luteinizing hormone
LH-R	luteinizing hormone receptor
LiCl	lithium chloride
LMP	low malignant potential

LOH	loss of heterozygosity
M	molar
MAb	monoclonal antibody
M-CSF	macrophage-colony stimulating factor
mg	milligram
mGC	murine granulosa cells
MgCl ₂	magnesium chloride
MGF	mast cell growth factor
min	minute
mJ	millijoule
ml	millilitre
ml/l	millilitre per litre
mm	millimetre
mM	millimolar
MnCl ₂	manganese chloride
mRNA	messenger ribonucleic acid
NaCl	sodium chloride
NaF	sodium fluoride
NaHCO ₃	sodium bicarbonate
Na ₂ HPO ₄	disodium hydrogen orthophosphate
NaH ₂ PO ₄	sodium dihydrogen orthophosphate
NaPP	sodium pyrophosphate
NCS	newborn calf serum
NEAA	non-essential amino acid solution
ng	nanogram
ng/μl	nanogram per microlitre
ng/ml	nanogram per millilitre
(NH ₄) ₃ VO ₄	sodium orthovanadate
nm	nanometre
oligo	oligonucleotide
OSE	ovarian surface epithelium
<i>p</i>	probability
P ₄	progesterone
pRB	retinoblastoma protein
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
pgk	phosphoglycerate kinase
PKA	protein kinase A

PKC	protein kinase C
pmol	picomole
PMSF	phenylmethyl sulfonyl fluoride
PR	progesterone receptor
PTP	protein tyrosine phosphatase
PY	phosphotyrosine
RNA	ribonucleic acid
rpm	revolutions per minute
RNase	ribonuclease
RPMI	RPMI Medium 1640
RT-PCR	reverse transcription-polymerase chain reaction
SCF	stem cell factor
SDS	sodium dodecyl sulphate
sec	second
SEM	standard error of the mean
s-KIT	soluble KIT
S-PBS	Stockholm phosphate-buffered saline
SSC	standard saline citrate
<i>Sl</i>	<i>Steel</i>
T	tumor
TAA	tumor-associated antigen
TAE	Tris-acetate, EDTA buffer
TBST	Tris-buffered, sodium chloride, Tween-20
TE	Tris-EDTA, pH 8.0
TGF- α	transforming growth factor-alpha
TGF- β	TGF-beta
Tris-HCl	pH-adjusted tris(hydroxymethyl)aminomethane
TVU	transvaginal ultrasonography
Tyr	tyrosine
U	unit
μ Ci	microcurie
μ Ci/ml	microcurie per millilitre
μ g	microgram
μ g/ml	microgram per millilitre
μ l	microlitre
μ m	micron
μ M	micromolar
v/v	volume per volume

w/v

weight per volume

X-gal

5-bromo-4-chloro-3-indolyl-B-D-galactopyranoside

Chapter 1 - Literature Review and Project Rationale

1.1 Ovarian Cancer: Overview

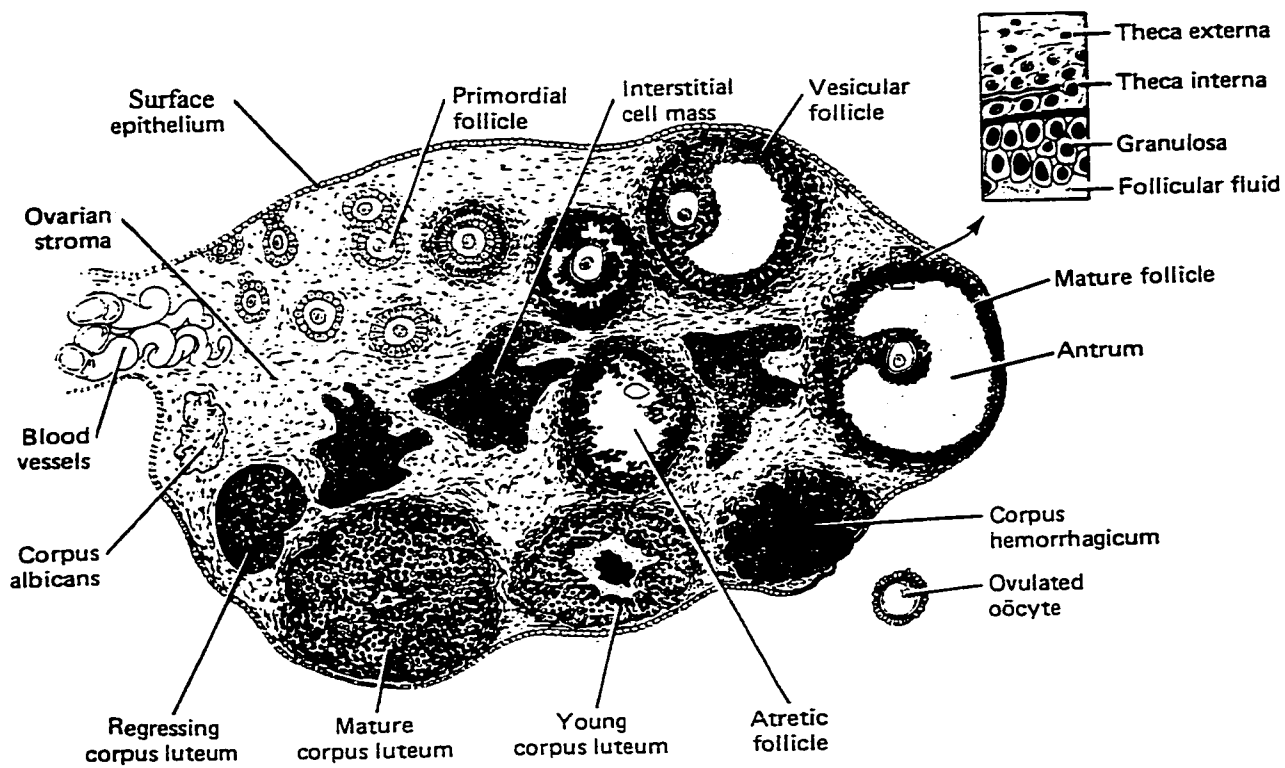
Ovarian cancer requires more research. Ovarian cancer is the fourth leading cause of all female cancer-related deaths in the Western World, and it is *the* most deadly cancer of all of the gynecological cancers. The disease carries a 1-in-70 lifetime risk for women in the Western World (Murdoch, 1996), and it is estimated that a staggering 60% of the Canadian women who develop ovarian cancer in the year 2000 will die from their disease (National Cancer Institute of Canada, 2000). Despite improved knowledge of the etiology of the disease, aggressive cytoreductive surgery, and modern combination chemotherapy, there has been little change in the mortality statistics over the last 20 years (National Cancer Institute of Canada, 2000). Although ovarian cancer can arise from any of the cell types found in the ovary (Figure 1), including oocytes, granulosa cells, theca-interstitial cells and the ovarian surface epithelium (OSE), greater than 90% of ovarian cancers are derived from the OSE (Weiss *et al.*, 1977). Epithelial ovarian cancer is, therefore, the focus of the majority of ovarian cancer studies including this Ph.D. project.

1.2 Characterization of the Normal OSE

The OSE covers the entire ovarian surface, and varies morphologically from simple squamous to cuboidal to low pseudostratified columnar (Papadaki and Beilby, 1971; Blaustein and Lee, 1979). Embryologically derived from the mesodermal epithelium of the gonadal ridges, the OSE is continuous with the flattened mesothelium of the peritoneum

Figure 1

Diagram of a mammalian ovary. The sequential development of the follicle, formation of the corpus luteum, and follicular atresia are shown. A section of the wall of a mature antral follicle is enlarged at the upper right. Modified from Ganong (1981).



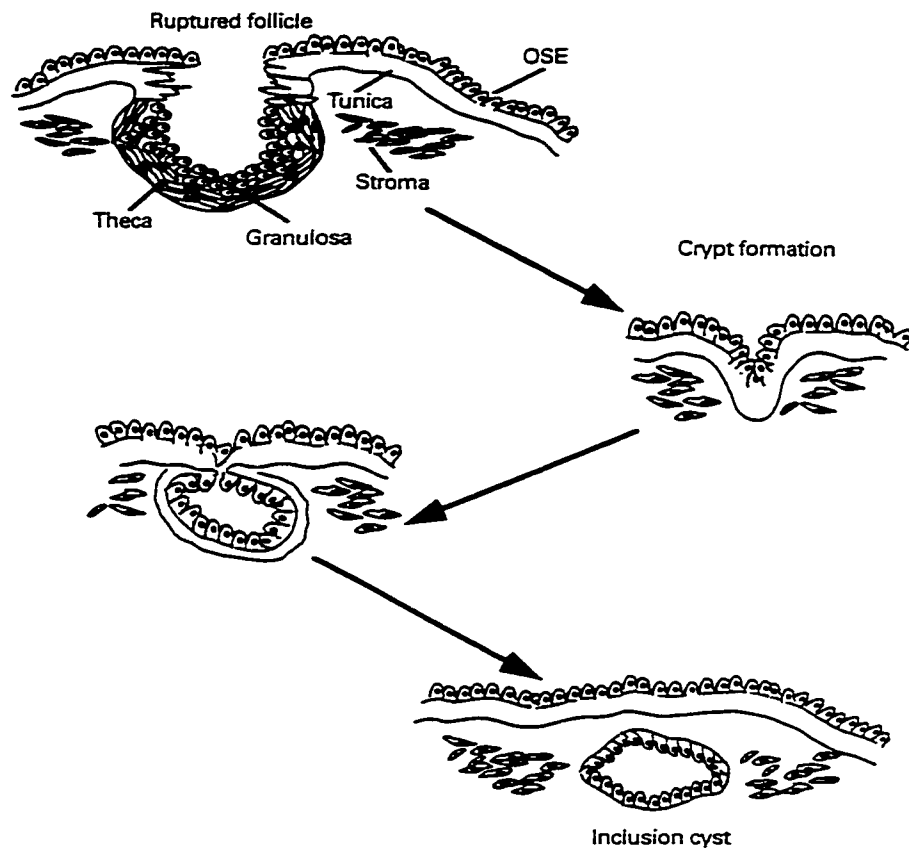
(Moore, 1992). The OSE is separated from the tunica albuginea of the ovarian cortex by a basement membrane, and the cells are joined laterally by desmosomes and gap and tight junctions (Espey, 1967; Ribeiro *et al.*, 1983). The cells have numerous apical microvilli and endocytic vesicles that allow for ready absorption of fluid and particulate matter (Perez *et al.*, 1991).

Despite their rather bland appearance *in vivo*, it is believed that OSE cells actively participate in the ovulatory process. Proteolytic enzymes released from cytoplasmic granules of OSE can apparently degrade the tunica albuginea and underlying follicular theca, thereby weakening the ovarian surface to the point of rupture (Bjersing and Cajander, 1974). The OSE cells directly over the point of rupture undergo apoptosis and are shed from the ovarian surface before ovulation (Ackerman and Murdoch, 1993). The wound created at the ovarian surface is repaired by rapid proliferation of OSE cells from the perimeter of the ruptured follicle (Osterholzer *et al.*, 1985a). Furthermore, the OSE cells are capable of depositing and restructuring the extracellular matrix of the underlying tunica albuginea following ovulation (Kruk and Auersperg, 1992; Kruk *et al.*, 1994).

OSE-lined inclusion cysts are believed to arise from the invagination and trapping of the OSE, which becomes sealed off to form inclusion cysts in the ovarian stroma (Figure 2). This may occur following the post-ovulatory proliferation of OSE, follicular attrition, or from inflammation caused by carcinogens or chemical irritants like talc (Hamilton, 1992). Although inclusion cysts are common in women and generally benign in nature, they are widely held to be the origin of many epithelial cancers, following early malignant changes. For example, some microscopic tumors of low malignant potential (LMP) and malignant

Figure 2

Inclusion cyst formation in the mammalian ovary. Following ovulation the wound on the ovarian surface is repaired by the proliferation of ovarian surface epithelial (OSE) cells. The healing process can lead to the formation of crypts or closed OSE-lined structures. Penetration of the tunica albuginea results in the formation of inclusion cysts that are in direct contact with stromal cells. Taken from Ghahremani *et al.* (1999).



tumors have been observed to arise directly within these sites, and they are often associated with dysplasia in similar sites elsewhere in the same or contralateral ovary (Deligdisch and Gil, 1989; Bell and Scully, 1994; Scully, 1995). Additionally, tubal metaplasia of epithelial inclusion cysts in ovaries contralateral to those containing epithelial ovarian cancers is significantly more common than in control ovaries, suggesting that Mullerian differentiation may be an early step in tumorigenesis (Mittal *et al.*, 1993; Resta *et al.*, 1993; Bell and Scully, 1994).

1.3 Classification of Epithelial Ovarian Cancers

Ovarian surface epithelium-derived ovarian cancers display a striking variety of morphological phenotypes, most of which recapitulate features of the normal genital and urologic tissues. In fact, the OSE and the urogenital tract share a common mesodermal origin, both being derived from the coelomic epithelium (Murdoch, 1996), and investigators have shown that OSE cells, in contrast to other adult epithelia, are relatively uncommitted and pluripotent (Dyck *et al.*, 1996). Epithelial ovarian cancers are histologically defined as serous, mucinous, endometrioid, or clear cell carcinomas based on their resemblance to the epithelia of the Fallopian tubes, endocervix, endometrium, or mesonephros, respectively; additional morphologies are Brenner (transitional), mixed epithelial, undifferentiated and unclassifiable (Anderson *et al.*, 1985). Close to 50% of all ovarian carcinomas are of serous histopathology (Ozols *et al.*, 1992), while clear cell tumors are the most lethal subtype and account for approximately 10% of the tumors (Talerman, 1992).

Clinically, tumor behavior is classified as being benign, LMP (borderline invasive),

or malignant (invasive). LMP and malignant tumors are also assigned a grade, which indicates how closely the tumor cells resemble the normal tissue counterparts. Grade 1 refers to a well differentiated tumor, grade 2 is a moderately differentiated tumor, while grade 3 is a poorly differentiated tumor.

Ovarian cancers are further categorized by stage to indicate disease progression, i.e., how far the tumor cells have disseminated from their site of origin. Four stages of disease have been defined by the International Federation of Gynecology and Obstetrics (FIGO). Stage I cancer is found in one (Ia) or both (Ib) ovaries, and is further defined by OSE-lined inclusion cysts which invade the ovarian cortex. Prognosis is good if the tumor growth has remained limited internally or the cyst(s) has not ruptured. A further subclass of stage I describes the extrusion of malignant cells from a ruptured inclusion cyst into the peritoneal cavity (Ic). In stage II disease, the tumor involves one or both ovaries with pelvic extension to the uterus and/or Fallopian tubes (IIa), or other pelvic tissues (IIb). The presence of ascites or peritoneal washings containing malignant cells means a stage IIc classification. Stage III disease involves one or both ovaries with microscopic peritoneal metastasis outside the pelvis (IIIa); stage IIIb has macroscopic peritoneal metastasis ≤ 2 cm in diameter beyond the pelvis; and stage IIIc has peritoneal metastasis > 2 cm in diameter beyond the pelvis and/or regional lymph node metastasis. Superficial liver metastasis and malignant extension to the small bowel or omentum are typical of invasive stage III disease. Stage IV disease has distant disseminated metastases to encompass pleural effusion and parenchymal liver (American Joint Committee on Cancer, 1992).

1.4 Symptomatology and Screening

Ovarian cancer is known as ‘the disease that whispers’ because, sadly, there is little specific symptomatology associated with early stage disease. Yet, early detection is the key to saving women’s lives, since there is a 90% cure rate by surgery (salpingo-ovariectomy) alone if the diagnosis is at stage I (Murdoch, 1996). According to the U.S. National Ovarian Cancer Coalition, symptoms of ovarian cancer include: pelvic or abdominal disturbances such as pain, discomfort, swelling, bloating, and/or a feeling of fullness; vague but persistent gastrointestinal upsets like nausea and indigestion; frequency and/or urgency of urination in the absence of an infection; unexplained changes in bowel habits; unexplained weight gain (particularly in the abdominal region) or weight loss; pain during sexual intercourse; ongoing fatigue; and, rarely, abnormal postmenopausal bleeding. Clearly, only pelvic/abdominal symptoms readily hint of ovarian cancer. Yet, increased abdominal size and pelvic pressure are generally indicative of ascites accumulation and widespread metastases of advanced stage disease (Murdoch, 1996); indeed, only 24% of all cases are detected when the disease is confined to just the ovary (Landis *et al.*, 1998).

To date, there is no single, reliable, specific screening method for detecting ovarian cancer. At a minimum, annual vaginal (women ≥ 18 years of age) and rectovaginal (women ≥ 35 years of age) examinations should be performed. Additionally, following appraisal of a woman’s risk factor assessment from medical, reproductive, and family cancer histories, there are currently 3 main screening procedures (Collins *et al.*, 1998): measurement of tumor-associated antigens (TAA); transvaginal ultrasonography (TVU); and/or determination of *BRCA1* gene mutations (see section 1.5.3.2).

Tumor-associated antigens are serum biomarkers present on, or released by, tumor cells, which can be assayed using monoclonal antibodies (MAb). A number of TAAs have shown some promise as indicators of disease status, progression, and/or recurrence, including CA-125, TAG-72, OVX-1, M-CSF, and CA 15-3 (reviewed in Teneriello and Park, 1995). Of these, CA-125 (Bast *et al.*, 1983) is the most widely used ovarian cancer biomarker. Elevated levels of CA-125 (>35 U/ml) have been reported in 61-96% of all clinically diagnosed epithelial ovarian cancers (Carlson *et al.*, 1994). However, CA-125 measurement does have its limitations. CA-125 has a low detection rate for stage I tumors (Jacobs *et al.*, 1993). Furthermore, as with other TAAs, measurement of CA-125 can suffer from a lack of specificity: elevated CA-125 levels are also found in patients with non-ovarian malignancies, e.g., liver, pancreas, and endometrium, plus false positive results are common in pregnancy and occur in nonmalignant conditions such as pelvic inflammatory disease, uterine fibroids, endometriosis, peritonitis, pancreatitis, renal failure and alcoholic hepatitis (Murdoch, 1996; Collins *et al.*, 1998).

Measurement of multiple TAAs in the same sample has shown some improved efficacy over single TAA measurements for tumor detection. For example, co-measurement of CA-125 and OVX-I was shown to enhance the predictive value over CA-125 alone (Oram and Jeyarajah, 1994). Woolas *et al.* (1993) demonstrated that the levels of at least one of three serum antigens (CA 125, OVX-1, or M-CSF) were raised in 98% of 46 patients with clinically detected stage I ovarian cancer. Moreover, results from multiple serum TAA assays were superior to individual tests for discriminating between benign and malignant pelvic masses (Woolas *et al.*, 1995).

Transvaginal ultrasonography uses high-frequency sound waves to detect persistent changes in the size, shape and echogenicity of one or both ovaries, and color Doppler imaging can be used in conjunction with TVU to assess intratumoral vascularity (Collins *et al.*, 1998). TVU is especially useful for women at high risk of developing ovarian cancer, and it has been beneficial in identifying asymptomatic women with stage I lesions (Oram and Jeyarajah, 1994). Thus, it has been proposed that a multimodal approach employing a panel of tumor biomarkers as a primary screening test with TVU as a secondary test could be a reasonable, cost-effective strategy (Murdoch, 1996).

1.5 The Etiology of Epithelial Ovarian Cancer

There is evidence that epithelial ovarian cancer arises by clonal expansion from a single transformed progenitor cell (Jacobs *et al.*, 1992; Mok *et al.*, 1992). Yet, a definitive mechanism or series of events *commonly* leading to the malignant transformation of OSE cells has not been elucidated. Nevertheless, there are a number of factors which have shown some physiological relevance in epithelial ovarian tumorigenesis and patient prognosis, and these will be described under the categories of (1) ovulation, (2) endocrine factors, (3) genetics, (4) growth factors and cytokines, and (5) environmental carcinogens and infectious agents.

1.5.1 Ovulation

The “incessant ovulation hypothesis” proposed that continuous ovulation, with its successive bouts of surface rupture and OSE cell mitosis to repair the wound, drives

malignant transformation of the OSE (Fathalla, 1971). Anecdotal support for this hypothesis came from the finding that intensive egg-laying domestic hens frequently develop peritoneal carcinomata that is presumably of ovarian origin (Fredrickson, 1987). Epidemiological studies indicate that a range of circumstances which decrease the number of ovulations, i.e., pregnancy, oral contraceptive usage, and duration of lactation, all substantially reduce the risk of ovarian cancer (Whittemore *et al.*, 1992; La Vecchia and Franceschi, 1999). Furthermore, although still controversial, some investigators have found an increased risk of developing ovarian cancer among women treated with ovulation induction drugs in assisted reproduction programs (reviewed in Burmeister and Healy, 1998). Experimental support for Fathalla's hypothesis came from studies showing that primary cultures of normal rat OSE cells which had been repeatedly subcultured to mimic incessant ovulation acquired features associated with malignant transformation, including loss of contact inhibition, substrate-independent growth, and the ability to form tumors in nude mice (Godwin *et al.*, 1992a).

1.5.2 Endocrine Factors

A second explanation, known as the “gonadotropin secretion theory”, proposed that the development of ovarian cancer is related to excessive gonadotropin production associated with the onset of menopause or premature ovarian failure (Cramer and Welch, 1983). The median age for epithelial ovarian cancer is 60-65 years, with only 10-15% of the tumors appearing in premenopausal women (Sell *et al.*, 1990). Elevated gonadotropin levels are due to an age-related decreased sensitivity, at the level of the hypothalamus-pituitary, to negative

feedback normally mediated by follicle-produced estrogen (reviewed in Richardson, 1993). In fact, both gonadotropic and steroid hormones of the hypothalamic-pituitary-ovarian axis have been implicated in ovarian tumorigenesis, including gonadotropin-releasing hormone (GnRH), follicle-stimulating hormone (FSH), luteinizing hormone (LH)/human chorionic gonadotropin (hCG), estrogen (E), progesterone (P₄), and androgen.

Normal human OSE cells have been found to express both GnRH and its receptor, and treatment of the cells with GnRH analogs caused decreased proliferation (Kang *et al.*, 2000). Similarly, treatment with GnRH agonists has been shown to inhibit the growth of ovarian carcinoma cells (Kimura *et al.*, 1999; Kim *et al.*, 1999) and human ovarian tumor xenografts in nude mice (Peterson *et al.*, 1994; Manetta *et al.*, 1995; Kim *et al.*, 1999). In ovarian cancer cell lines, decreased proliferation in response to GnRH analogs was associated with a G0/G1 phase block in the cell cycle (Kim *et al.*, 1999) and hypophosphorylation of the retinoblastoma protein, pRB (Kimura *et al.*, 1999). Conversely, Yano *et al.* (1994) have reported that treatment of athymic mice bearing human epithelial ovarian cancer xenografts with a GnRH antagonist resulted in decreased tumor growth. Perhaps not surprisingly, then, clinical studies using a single GnRH agonist for treatment of advanced ovarian cancer and in combination therapy with cisplatin have demonstrated conflicting results (Van der Vange *et al.*, 1995; Emons *et al.*, 1996; Falkson *et al.*, 1996).

Normal human OSE cells and epithelial inclusions have been found to express receptors for FSH (FSH-R; Zheng *et al.*, 1996) and LH/hCG (LH-R; Konishi *et al.*, 1999). Enhanced cell proliferation in response to FSH and/or LH/hCG occurred in primary rabbit (Osterholzer *et al.*, 1985b) and human (Konishi *et al.*, 1999) OSE cells. Indeed, treatment

of primary human OSE cells with hCG could prevent apoptosis induced by serum deprivation (Konishi *et al.*, 1999).

FSH-R and LH-R have been identified on benign and malignant ovarian tumor cells (Kammerman *et al.*, 1981; Kobayashi *et al.*, 1996; Mandai *et al.*, 1997) and on ovarian carcinoma cells (Emons *et al.*, 1992; Godwin *et al.*, 1992b; Mandai *et al.*, 1997). Enhanced cell proliferation in response to FSH and/or LH/hCG treatment has been demonstrated for primary ovarian cancer cell cultures (Wimalasena *et al.*, 1992; Kurbacher *et al.*, 1995) and ovarian carcinoma cell lines (Simon *et al.*, 1983; Wimalasena *et al.*, 1992). In one report, hCG did not affect the proliferation of two ovarian carcinoma cell lines, but its addition significantly suppressed cisplatin-induced apoptosis in one of the lines (Kuroda *et al.*, 1998). Additionally, Schiffenbauer *et al.* (1997) found that human epithelial ovarian cancers progressed faster in ovariectomized mice due to the animals' elevated FSH and LH levels, which promoted increased vascular endothelial growth factor expression and tumor neovascularization. Interestingly, Zheng *et al.* (2000) recently found that the growth of two FSH-R- and LH-R-expressing ovarian carcinoma cell lines was stimulated by FSH but not by LH, and that the FSH-stimulated growth was actually opposed by simultaneous treatment with LH, suggesting a reason why high postmenopausal gonadotropin levels or ovulation induction drugs do not always correlate with increased ovarian cancer risk.

Thus, numerous studies have suggested that gonadotropins may support ovarian tumorigenesis. Most recently, Zheng *et al.* (2000) found FSH-R expression in 100% of epithelial inclusions (n=20) and benign tumors (n=12), 94% of LMP tumors (n=18), but only 60% of the carcinomas (n=30), suggesting that FSH may be more important in early

tumorigenesis.

In the developing fetal ovary, marked OSE cell proliferation occurs at 16 to 20 weeks of gestation, coincident with the appearance of steroid-producing cells in the ovarian cortex (Gondos, 1975). Adult human OSE cells do express receptors for estrogen (ER), progesterone (PR), and androgen (AR; Karlan *et al.*, 1995; Lau *et al.*, 1999), and human OSE cell proliferation can be stimulated by androgens (Hamilton *et al.*, 1983), but is unaffected by E or P₄ (Karlan *et al.*, 1995). Indeed, testosterone-stimulated growth of OSE cells in guinea pigs caused the formation of benign epithelial ovarian neoplasms (Silva *et al.*, 1997).

ER, PR, and AR have been detected, with varying levels of expression and activity, in many ovarian tumors (Kommos *et al.*, 1992; Kobayashi *et al.*, 1996; Brandenberger *et al.*, 1998) and ovarian carcinoma cell lines (Hamilton *et al.*, 1983; Kuhnel *et al.*, 1987; Grenman *et al.*, 1994; Kobayashi *et al.*, 1996; Baldwin *et al.*, 1998). Although there does not appear to be any correlation between the number of ER or PR and tumor stage (Vihko *et al.*, 1983), ER expression has been reported to be higher in well differentiated malignant tumors (Iversen *et al.*, 1986), and less highly expressed in metastatic tumors relative to the primary tumor (Quinn *et al.*, 1988). Interestingly, high tumor ER and PR positivity has been associated with better patient survival in some cases (Slotman *et al.*, 1990; Kieback *et al.*, 1993).

Heinonen *et al.* (1985) measured elevated levels of progesterone in patients with epithelial ovarian cancer, particularly in those patients with mucinous tumors. Indeed, endogenous steroid production by ovarian tumors has been documented (Abrahamsson *et al.*, 1997). Treatment with E or P₄ has been shown to stimulate proliferation of many ovarian

carcinoma cell lines (Nash *et al.*, 1989; Langdon *et al.*, 1990; Langdon *et al.*, 1993; Wimalasena *et al.*, 1993; Langdon *et al.*, 1994; Ridderheim *et al.*, 1994; Simpson *et al.*, 1998; Baldwin *et al.*, 1998). In some ovarian carcinoma cells, the mitogenicity in response to E and P₄ has been associated with increased transforming growth factor- α (TGF- α) production (Ridderheim *et al.*, 1994; Simpson *et al.*, 1998; see section 1.5.4), and rapid transcriptional induction of *c-myc* (Chien *et al.*, 1994; see section 1.5.3.1). ER binding to the estrogen response element in the *H-ras* gene (see section 1.5.3.1) has also been demonstrated in ovarian tumors (Zachos *et al.*, 1996). Growth suppression in response to anti-progestins has been reported for PR-positive ovarian carcinoma cells (Parthasarathy *et al.*, 1996; Rose and Barnea, 1996). However, exposure of some ovarian cancer cell lines to E or P₄ has resulted in anti-proliferative effects, including apoptosis and upregulation of the tumor suppressor gene *p53* (Wimalasena *et al.*, 1992; Bu *et al.*, 1997; see section 1.5.3.2), suggesting that hormonal anti-tumor therapies would be contraindicated in some patients. Nevertheless, modest patient response rates to synthetic progestins, anti-estrogens (e.g., tamoxifen) and anti-androgens have been reported (Rao and Slotman, 1991). Some studies have found that postmenopausal women who have taken steroid hormone replacement therapy may also be at a slightly increased risk of ovarian cancer (Whittemore *et al.*, 1992).

Although 50-60% of ovarian tumors express ER and/or PR, up to 95% of tumors express AR (Kuhnel *et al.*, 1987; Chadha *et al.*, 1993; Ilekis *et al.*, 1997), and androgens are the main steroids produced by the postmenopausal ovary (Adashi, 1994). An increased risk of ovarian cancer was found in women with elevated circulating levels of androgens (Helzlsouer *et al.*, 1995). Polycystic ovarian syndrome, a disease that is characterized in part

by elevated circulating levels of androgens, has also been associated with an increased risk of ovarian cancer (Schildkraut *et al.*, 1996). The proliferation of several ovarian carcinoma cell lines has been inhibited by anti-androgens (Hamilton *et al.*, 1983; Slotman and Rao, 1989). In support of these findings, Evangelou *et al.* (2000) demonstrated that treatment of ovarian carcinoma cells with the androgen 5 α -dihydrotestosterone caused downregulation of transforming growth factor- β (TGF- β) receptors, thus, androgens may promote ovarian cancer progression in part by decreasing TGF- β receptor levels, thereby allowing ovarian cancer cells to escape TGF- β growth inhibition (see section 1.5.4).

Thus, it appears that only androgens may contribute to early ovarian tumorigenesis by regulating OSE cell growth, while all of the steroid hormones could be functioning to promote ovarian tumor growth.

1.5.3 Genetics

Many ovarian cancers exhibit aneuploidy and many studies have shown that ploidy is an independent prognostic variable for patient survival (reviewed in Friedlander, 1998). More specifically, genetic alterations involving oncogenes and tumor suppressor genes, that have critical roles in normal cell growth, have been found to be important in ovarian tumorigenesis. Although many potential candidates have been studied in this regard (reviewed in Auersperg *et al.*, 1998 and Lynch *et al.*, 1998), only the best characterized genes will be discussed here.

1.5.3.1 Oncogenes

The three members of the *ras* oncogene family, H-, K-, and N-*ras*, encode p21^{ras} G proteins that are involved in promoting cell growth. K-*ras* mutations have correlated most frequently with the mucinous ovarian tumor subtype, having been detected in 11-75% of mucinous versus 5-36% of non-mucinous tumors (reviewed in Aunoble *et al.*, 2000). Tumor cells with *ras* mutations demonstrated resistance to chemotherapy and radiotherapy (Sklar, 1988a; Sklar, 1988b), suggesting a reason why K-*ras* mutation in human ovarian tumors correlated with a shorter patient survival in one study (Scambia *et al.*, 1997). Intriguingly, stable expression of the H-*ras* oncogene in immortalized rat OSE cells (ROSE 199 cells) resulted in malignant transformation of the cells, as evidenced by loss of contact inhibition, growth in soft agar *in vitro*, and the ability to form invasive tumors when injected subcutaneously in immunocompetent rats; however, these *ras*-transformed OSE cells generated sarcomas rather than tumors resembling ovarian carcinomas (Hoffman *et al.*, 1993).

The HER-2/*neu* gene encodes a transmembrane glycoprotein with tyrosine kinase activity which belongs to a family of growth factor receptors that includes the epidermal growth factor (EGF) receptor (EGF-R; see section 1.5.4). Normal human OSE cells express little or no HER-2/*neu* (Berchuck *et al.*, 1990; Gordon *et al.*, 1995), but amplification and/or over-expression of HER-2/*neu* has been reported in 19-59% of epithelial ovarian cancers (reviewed in Aunoble *et al.*, 2000) and some ovarian carcinoma cell lines (King *et al.*, 1992). No relationship between HER-2/*neu* amplification and histologic tumor grade has been found (Zheng *et al.*, 1991), but a correlation between HER-2/*neu* over-expression and poor

prognosis has been reported in approximately half of the studies (reviewed in Aunoble *et al.*, 2000). Indeed, in other cell systems, HER-2/*neu* over-expression has been associated with taxol resistance (Yu *et al.*, 1996) and metastasis (Yu *et al.*, 1994). Notably, over-expression of activated HER-2/*neu* in ROSE 199 cells resulted in highly tumorigenic cells that grew as solid tumors when injected into immunocompetent rat ovaries, with peritoneal seeding and ascites formation; however, like the *ras*-transformed ROSE 199 cells, ROSE 199-*neu* tumors possessed sarcomatous morphology (Davies *et al.*, 1998).

The *c-myc* oncogene encodes a transcription factor that regulates cell proliferation via effects on the cell cycle. Amplification and over-expression of the *c-MYC* gene has been detected in ovarian cancer cell lines (Yasue *et al.*, 1987) and in 26-37% of epithelial ovarian cancers (reviewed in Aunoble *et al.*, 2000). *C-MYC* over-expression is more common in malignant serous ovarian tumors and is associated with stage III disease, suggesting a role in disease progression (Tashiro *et al.*, 1992). Although there has been no correlation observed between *c-myc* over-expression and patient survival (reviewed in Aunoble *et al.*, 2000), one study did demonstrate poor prognosis if *c-myc* over-expression was associated with high levels of HER-2/*neu* and p21^{ras} (Katsaros *et al.*, 1995).

1.5.3.2 Tumor Suppressor Genes

The most widely used approach to identify where tumor suppressor genes may reside is by examination for loss of heterozygosity (LOH). Studies have shown LOH for chromosomes 1p, 3p, 4p, 5q, 6q, 7p, 7q, 8q, 9p, 11p, 11q, 12p, 12q, 13q, 14q, 16p, 16q, 17p, 17q, 18q, 19p, 22q, Xp, and Xq in ovarian tumor specimens (reviewed in Auersperg *et al.*,

1998 and Lynch *et al.*, 1998). Although the identification of most of these genetic loci remains to be determined, loss and/or inactivation of a few tumor suppressor genes have been correlated with patient survival.

To date, only one *inheritable* risk factor for developing epithelial ovarian cancer has been identified, namely mutation in the *BRCA1* or *BRCA2* genes found on chromosomes 17 and 13, respectively. The *BRCA1* protein is involved in DNA damage repair via interaction with RAD51 (Scully *et al.*, 1997a; Chen *et al.*, 1999), and *BRCA1* may also be involved in gene transcription since it can associate with RNA polymerase holoenzyme II (Scully *et al.*, 1997b). *BRCA1* is considered to be a tumor suppressor gene since retroviral infection of the gene into ovarian and breast cancer cells suppresses their growth *in vitro* and tumorigenicity *in vivo* (Holt *et al.*, 1996). Mutations in *BRCA1* are found in 34-58% of hereditary ovarian cancer families of North America and European origin (reviewed in Aunoble *et al.*, 2000). In general, mutations in the *BRCA1* gene account for approximately 78% of site-specific ovarian cancers and 92% of breast/ovarian cancer syndrome cases (Narod *et al.*, 1995). The risk among first degree relatives (i.e., mother, sister, daughter) of developing ovarian cancer can be as high as 50% (Murdoch, 1996). However, it is not clear whether *BRCA1* mutation actually affects ovarian cancer patient survival.

Although 80% of ovarian cancers show LOH at the *BRCA1* gene locus suggesting that inactivation of the *BRCA1* gene is related not only to familial ovarian cancer cases but also to sporadic cases, the estimated proportion of sporadic ovarian cancer due to *BRCA1* mutation is $\leq 5\%$ (Futreal *et al.*, 1994; Merajver *et al.*, 1995; Takahashi *et al.*, 1995).

The *BRCA2* protein is very similar in structure and function to *BRCA1*, and it is

estimated that 7-14% of ovarian cancer families harbor *BRCA2* mutations (reviewed in Aunoble *et al.*, 2000). In one study, *BRCA2* mutations were detected in 9% of 119 families, but the authors found no evidence for differences in survival of the women (Pharoah *et al.*, 1999). Like *BRCA1* gene mutations, *BRCA2* mutations occur in only about 5% of sporadic ovarian cancer cases (Foster *et al.*, 1996; Takahashi *et al.*, 1996).

The *p53* tumor suppressor gene is the most frequently mutated gene in human neoplasms. The *p53* protein is a transcription factor that regulates the cell cycle via transactivation of genes involved in cell cycle arrest or apoptosis. Mutations and/or over-expression of *p53* have been described in 26-62% of ovarian cancers, particularly serous ovarian carcinomas (reviewed in Aunoble *et al.*, 2000). A genomic polymorphism of the *p53* gene that contains a 16 bp insertion in intron 3 (called PIN3) has been found to be associated with an increased risk of developing ovarian cancer (Runnebaum *et al.*, 1995). A correlation between *p53* alteration and poor patient survival has been reported in about 50% of the studies (reviewed in Aunoble *et al.*, 2000). Indeed, adenovirus-mediated gene transfer of wildtype *p53* into ovarian cancer cells containing a mutated *p53* gene has been shown to inhibit tumor growth (Santoso *et al.*, 1995; Mujoo *et al.*, 1996), and enhance sensitivity to chemotherapy and radiotherapy resulting in increased apoptosis (Gallardo *et al.*, 1996; Nielsen *et al.*, 1998).

Thus, molecular biological studies are providing valuable information regarding alterations in these, and other, genes in the hopes of identifying genetic models of epithelial ovarian cancers that could be exploited in the diagnosis and treatment of ovarian cancer. Indeed, a number of Phase I gene therapy clinical trials have been initiated to restore

wildtype *p53* or *BRCA1* gene function or to downregulate HER-2/*neu* over-expression (Tong *et al.*, 1999).

1.5.4 Growth Factors & Cytokines

There are a number of growth factors and cytokines which have been shown to regulate the proliferation of both normal human OSE cells and ovarian cancer cells and, thus, have been implicated in epithelial ovarian tumorigenesis.

Normal OSE cells express EGF-R (Berchuck *et al.*, 1991) and EGF has been shown to be a potent mitogen for these cells in tissue culture (Siemens and Auersperg, 1988; Rodriguez *et al.*, 1991). TGF- α , which resembles EGF structurally and functionally and can bind to and activate the EGF-R (Salomon *et al.*, 1990), is also produced by normal human OSE cells and their proliferation is stimulated by TGF- α (Jindal *et al.*, 1994). Like normal OSE cells, the majority of ovarian carcinoma cells are also stimulated to proliferate in the presence of either EGF or TGF- α (Stromberg *et al.*, 1992; Berchuck *et al.*, 1992; Wimalasena *et al.*, 1992; Jindal *et al.*, 1994; Simpson *et al.*, 1998), and the growth of ovarian carcinoma cells in serum-free tissue culture can be inhibited by treatment with anti-TGF- α neutralizing antibodies (Stromberg *et al.*, 1992). EGF has been shown to cause a significant reduction in the levels of HER-2/*neu* protein in ovarian carcinoma cells (Marth *et al.*, 1992), while TGF- α -stimulated growth of ovarian carcinoma cells has been associated with upregulation of its own mRNA expression (Jindal *et al.*, 1995) and *c-myc* gene expression (Park, 1997). Recently, though, tumor EGF-R status was found to have no independent prognostic or predictive significance in advanced disease (n=185; Baekelandt *et al.*, 1999).

TGF- β is a powerful growth inhibitor in many cell types, including epithelial cells (Lyons and Moses, 1990). Normal human OSE cells produce TGF- β which does act as an autocrine growth inhibitor (Berchuck *et al.*, 1992; Havrilesky *et al.*, 1995). The proliferation of various ovarian cancer cells has also been demonstrated to be inhibited by exogenous TGF- β , including primary ovarian cancer cells from solid tumors and patients' ascites (Hurteau *et al.*, 1994; Havrilesky *et al.*, 1995), as well as ovarian carcinoma cell lines (Berchuck *et al.*, 1992; Kim *et al.*, 1997). Many of these ovarian cancer cells also produce endogenous TGF- β (Berchuck *et al.*, 1992; Hurteau *et al.*, 1994). In conjunction with its growth suppression of ovarian cancer cells, TGF- β has been shown to induce apoptosis (Havrilesky *et al.*, 1995; Lafon *et al.*, 1996), and to downregulate expression of TGF- α (Jindal *et al.*, 1995), *c-myc* (Kim *et al.*, 1997) and the anti-apoptotic gene *bcl-2* (Lafon *et al.*, 1996). However, it should be noted that some ovarian cancer cells were resistant to the growth inhibitory effects of TGF- β and/or did not produce TGF- β (Berchuck *et al.*, 1992; Hurteau *et al.*, 1994), pointing to a method of escaping the negative growth regulation of TGF- β during tumor progression.

Normal OSE cells also secrete bioactive cytokines including interleukin (IL)-1 and IL-6, granulocyte-colony stimulating factor (G-CSF), macrophage (M)-CSF, and GM-CSF (Ziltener *et al.*, 1993). With the exception of G-CSF, all of these factors are also produced by ovarian cancer cells and stimulate their proliferation (Berchuck *et al.*, 1993). The M-CSF receptor (encoded by the *c-FMS* protooncogene; see section 1.7) is expressed by many malignant ovarian cancers but not by benign tumors (Kacinski *et al.*, 1995) or normal OSE cells (Berchuck *et al.*, 1993), suggesting that M-CSF secreted by the normal OSE may act

in a paracrine manner but subsequently become an autocrine regulatory factor with malignant progression. Indeed, the ability of ovarian cancer cells to invade has been correlated with high endogenous M-CSF production (Chambers *et al.*, 1995).

1.5.5 Environmental Carcinogens and Infectious Agents

Weak correlations have been made between exposure to talc/asbestos (that presumably ascends the genital tract and affects the ovarian surface), alcohol and coffee usage, and high fat diets and increased risk of developing ovarian cancer (Shu *et al.*, 1989; Gertig *et al.*, 2000). Infections that may lead to premature ovarian failure (e.g., mumps virus) have been related to increased ovarian cancer risk (Menczer *et al.*, 1979), and it has been proposed that sexually transmitted disease organisms may underlie the increased risk associated with infertility (Hardiman *et al.*, 1999).

1.6 Malignant Transformation of Normal OSE

Despite all of this knowledge pertaining to the etiology of epithelial ovarian cancer, a precise sequence of events, particularly early events, resulting in malignant transformation of OSE cells is uncertain. In this regard, it may be that an increased commitment to an epithelial phenotype is one of the earliest changes in the process of ovarian tumorigenesis. Normal OSE cells express both epithelial (e.g., keratin) and mesenchymal (e.g., collagen type III) markers *in vivo* (Czernobilsky *et al.*, 1985; van Niekerk *et al.*, 1993) and *in vitro* (Auersperg *et al.*, 1984; van Niekerk *et al.*, 1989), but in OSE-lined inclusion cysts epithelial differentiation predominates, often in conjunction with Mullerian metaplasia (Salazar *et al.*,

1996). Indeed, ovarian cancers are distinctly epithelial with expression of keratin, CA-125, epithelial (E)-cadherin, cilia, and the formation of papillae and glands (van Niekerk *et al.*, 1993; Sundfeldt *et al.*, 1997; Maines-Bandiera and Auersperg, 1997); in primary culture, ovarian cancer cells retain this epithelial phenotype (Auersperg *et al.*, 1994). Interestingly, OSE cells cultured from the ovaries of women with strong family histories of ovarian cancer contained more CA-125-expressing cells, more keratin, more E-cadherin, and less collagen type III compared with overtly normal OSE cells from patients with no family history (Auersperg *et al.*, 1995; Dyck *et al.*, 1996; Wong *et al.*, 1999). Most recently, constitutive expression of E-cadherin in SV40-immortalized human OSE cells has been shown to induce epithelial differentiation (e.g., CA-125 expression; Auersperg *et al.*, 1999), and the cells formed adenocarcinomas when injected into SCID mice (Ong *et al.*, 1999), suggesting that there may be a causal relationship between E-cadherin expression and neoplastic progression of human OSE.

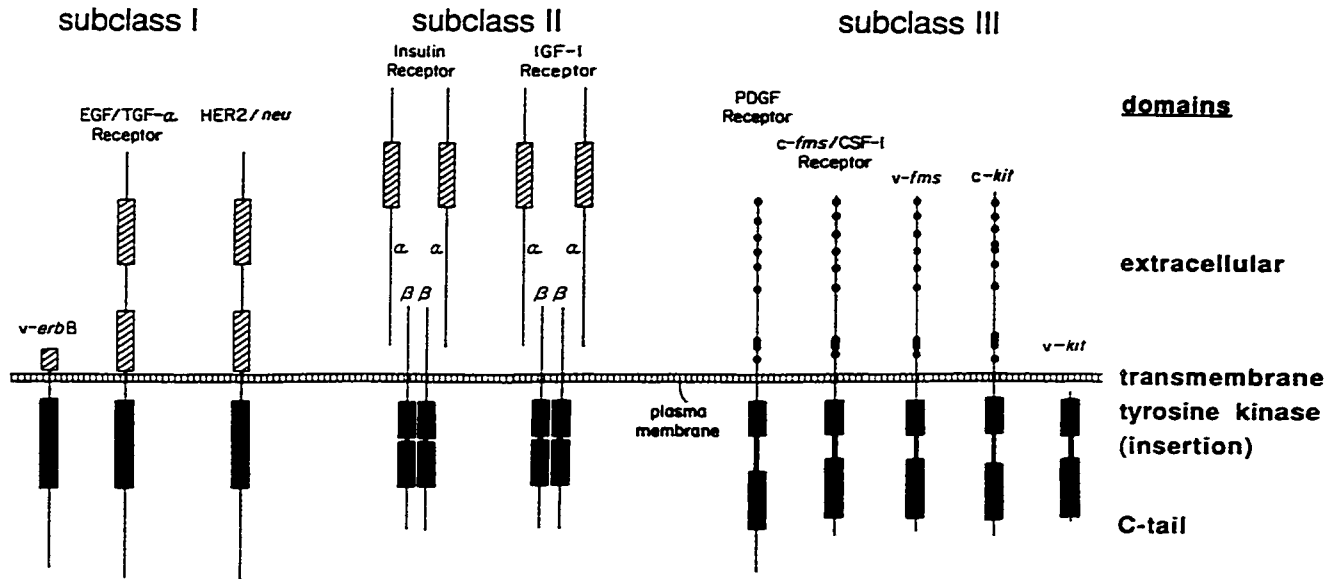
1.7 Molecular Biology of c-*KIT*

The c-*KIT* protooncogene is the normal cellular homologue of v-*kit*, the transforming gene of the Hardy-Zuckerman 4 strain of feline sarcoma virus (Besmer *et al.*, 1986; Yarden *et al.*, 1987). The *KIT* locus (formerly known as the *W*, for *White-spotting*, locus) maps to human chromosome 4q11-13 (Yarden *et al.*, 1987) and encodes KIT protein, a class III tyrosine kinase receptor which shares homology with the platelet-derived growth factor (PDGF) receptor and c-*FMS* receptor (Figure 3; Yarden *et al.*, 1987; Geissler *et al.*, 1988; Qiu *et al.*, 1988). These receptors are characterized by an extracellular domain containing

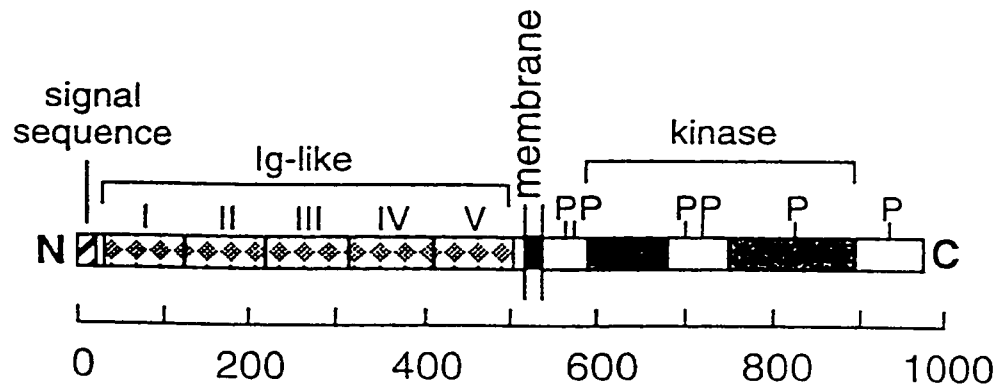
Figure 3

The KIT receptor. (A) Shown are the subclasses and structural features of the family of receptor tyrosine kinases (RTKs) and their known viral oncogenic counterparts. Hatched regions represent cysteine-rich repeat domains, closed boxes demarcate the tyrosine kinase domains, and closed circles indicate cysteine residues in the extracellular portions of subclass III RTKs. Taken from Yarden and Ullrich (1988). (B) Linear representation of the domain structure of KIT, showing the six potential tyrosine phosphorylation sites (P) in the cytoplasmic portion of the receptor. Taken from Hardie and Hanks (1995).

A



B



five immunoglobulin (Ig)-like repeats, a transmembrane domain, and an intracellular domain that possesses tyrosine kinase activity (Yarden *et al.*, 1987; Qiu *et al.*, 1988; Yarden and Ullrich, 1988).

The coding region of the *c-KIT* gene is distributed over 21 exons and generates a 5.1 kb transcript in human cells (Vandenbark *et al.*, 1992; Giebel *et al.*, 1992). Depending on the amount of posttranslational glycosylation, KIT proteins are 125, 145 and 160 kD in size (Yarden *et al.*, 1987; Reith *et al.*, 1991; Furitsu *et al.*, 1993; Matsuda *et al.*, 1993). Additionally, an alternatively spliced *c-KIT* isoform, arising through the use of an alternative splice-donor site in exon 9 (Vandenbark *et al.*, 1992; Giebel *et al.*, 1992), contains 12 additional bases that create a 4 amino acid proteolytic cleavage site in the juxtamembrane region of the KIT receptor (Reith *et al.*, 1991; Hayashi *et al.*, 1991; Giebel *et al.*, 1992; Gokkel *et al.*, 1992). Proteolysis releases the extracellular domain of KIT (Kawakita *et al.*, 1995; Turner *et al.*, 1995; Wypych *et al.*, 1995) as a 95 kD soluble KIT (s-KIT) protein (Broudy *et al.*, 1994; Kawakita *et al.*, 1995; Turner *et al.*, 1995). S-KIT has been found to circulate in human plasma at a concentration of 325 ± 105 ng/ml (Wypych *et al.*, 1995). The proteolytic processing which generates s-KIT is enhanced with protein kinase C (PKC) activation and increased intracellular calcium levels (Yee *et al.*, 1993; Yee *et al.*, 1994a). It is thought that s-KIT may act as an endogenous antagonist to KIT activity by competing with membrane-associated KIT for ligand (Turner *et al.*, 1995).

1.8 Molecular Biology of Stem Cell Factor (SCF), the Ligand to Kit

The ligand for the KIT receptor has been variously known as Kit ligand (Huang *et al.*,

1990), mast cell growth factor (MGF; Anderson *et al.*, 1990; Williams *et al.*, 1990), stem cell factor (SCF; Zsebo *et al.*, 1990; Martin *et al.*, 1990), or Steel factor (Witte, 1990), and will be referred to as SCF herein. SCF is encoded by the *Mgf* locus (formerly known as the *Sl*, for *Steel*, locus), which maps to human chromosome 12 (Anderson *et al.*, 1990; Geissler *et al.*, 1991). The major SCF transcript is generated from 9 exons and yields a predominant 6 kb mRNA transcript in human cells (Martin *et al.*, 1990). As is the case for c-*KIT*, there are two major splice variants of SCF resulting from an alternative splicing event that produces mRNA species that either include or exclude exon 6 (Anderson *et al.*, 1990; Flanagan *et al.*, 1991). The SCF protein arising from the larger mRNA is proteolytically cleaved in the extracellular domain to release a soluble SCF, while translation of the shorter SCF mRNA results in a protein that is more stably associated with the membrane (Flanagan *et al.*, 1991; Huang *et al.*, 1992). The extracellular portion of SCF is extensively glycosylated and, depending on its posttranslational modifications, SCF protein is 25-40 kD (Huang *et al.*, 1990; Arakawa *et al.*, 1991). Proteolytic cleavage of SCF from the cell surface can be induced by PKC or by agents that increase cytosolic calcium levels (Huang *et al.*, 1992), and can be prevented by serine protease inhibitors (Pandiella *et al.*, 1992). Soluble SCF has been found in human plasma at an average concentration of 3.3 ng/ml (Langley *et al.*, 1993), and it circulates as noncovalently-associated dimers (Arakawa *et al.*, 1991). Differential biological potency of the two SCF isoforms has been documented. For example, cell-cell contact is essential for the ability of SCF produced by Sertoli cells to stimulate mast cell Kit receptors (Tajima *et al.*, 1991). A specific role for membrane-associated SCF has also been demonstrated by the sterility of *Sl^d* mutant mice which express only soluble SCF (Nakayama

et al., 1988; Brannan *et al.*, 1991; Flanagan *et al.*, 1991).

1.9 Signal Transduction from the Activated KIT Receptor

The signal transduction events initiated upon SCF binding to KIT have been extensively studied in a variety of cell types, including mast cells (Reith *et al.*, 1991; Rottapel *et al.*, 1991), hematopoietic progenitor cells (Okuda *et al.*, 1992), myeloid cells (Miyazawa *et al.*, 1992), and transfected fibroblast cells (Herbst *et al.*, 1991; Lev *et al.*, 1991). Binding of SCF to Kit causes receptor dimerization and catalytic activation of the Kit kinase domain (Blume-Jensen *et al.*, 1991; Lev *et al.*, 1992) via phosphorylation of tyrosine (Tyr) 568 and Tyr570 (Lennartsson *et al.*, 1999). Three amino-terminal Ig-like motifs are involved in SCF binding to Kit, and the dimerization site resides in the fourth Ig-like motif which symmetrically interacts with its dimer partner (Blechman and Yarden, 1995).

Activated Kit receptor is known to associate with, phosphorylate, and/or activate a variety of downstream signaling molecules, including the p85 subunit of phosphatidylinositol 3'-kinase (Lev *et al.*, 1991; Rottapel *et al.*, 1991; Herbst *et al.*, 1992; Lev *et al.*, 1992; Blume-Jensen *et al.*, 1994; Serve *et al.*, 1994; Herbst *et al.*, 1995; Hines *et al.*, 1999), Janus Kinase 2 (Weiler *et al.*, 1996), p21^{ras} (Duronio *et al.*, 1992), p21^{ras}GTPase-activating protein (Herbst *et al.*, 1991; Herbst *et al.*, 1992), Raf-1 protein kinase (Herbst *et al.*, 1991; Lev *et al.*, 1991; Rottapel *et al.*, 1991), phospholipase C γ (Herbst *et al.*, 1991; Reith *et al.*, 1991; Rottapel *et al.*, 1991; Herbst *et al.*, 1992), the Grb2 adaptor protein, Src kinase (Blume-Jensen *et al.*, 1994), the Vav GDP/GTP exchange factor (Alai *et al.*, 1992), and members of the Ras/MAP kinase family (Okuda *et al.*, 1992; Tsai *et al.*, 1993; Blume-Jensen *et al.*, 1995;

Paulson *et al.*, 1996; Hines *et al.*, 1999). Additionally, Kit activation results in the recruitment and subsequent phosphorylation of two *src*-related tyrosine kinases, Tec (Tang *et al.*, 1994) and MATK (Jhun *et al.*, 1995), that are found only in hematopoietic cells.

Dephosphorylation of proteins at specific tyrosine residues by protein tyrosine phosphatases (PTP) has been shown to be essential for the normal regulation of growth factor activity. Kit receptor activity is negatively regulated, via distinct mechanisms, by a pair of PTPs known as SHP-1 and SHP-2 (Yi and Ihle, 1993; Feng and Pawson, 1994), and by PKC. Interaction of *src*-homology-2 domains present in SHP-1 and SHP-2 with phosphotyrosine (PY) residues of Kit causes dephosphorylation and, hence, inactivation of the Kit receptor (Feng and Pawson, 1994; Kozlowski *et al.*, 1998). PKC, on the other hand, inhibits Kit receptor autophosphorylation and kinase activity by phosphorylating activated serine residues on Kit (Blume-Jensen *et al.*, 1993). Interestingly, association of both SHP-1 and SHP-2 with Kit also results in the recruitment of the Grb2 adapter molecule (Yi and Ihle, 1993; Tauchi *et al.*, 1994) as well as activation of the Ras signaling pathway (Tauchi *et al.*, 1994; Kon-Kozlowski *et al.*, 1996).

Recently, another PTP present in megakaryocytes, called PTP-RO (for PTP-receptor omicron), has been shown to associate with, and become tyrosine phosphorylated, upon SCF binding to Kit (Taniguchi *et al.*, 1999).

1.10 Functional Relevance of the Kit:SCF Interaction in Normal Cells

Mutations at the *Kit* and *Mgf* (*W* and *Sl*) loci in mice profoundly affect the development of three migratory embryonic stem cell populations: neural crest-derived

melanocytes; hematopoietic precursors; and primordial germ cells (Russell, 1979). Numerous mutant alleles have been identified at these loci, and mice that are homozygous for severe mutations die during gestation or within days of birth, while viable heterozygous or homozygous mice carrying milder mutations exhibit varying degrees of white-spotting, erythrocyte and mast cell deficiencies, and infertility (reviewed in Morrison-Graham and Takahashi, 1993). The human homologue to the dominant white spotting phenotype of the mouse is piebaldism, a heterozygous condition characterized by a congenital white forehead lock and ventral and extremity depigmentation (Fleischman *et al.*, 1991; Giebel and Spritz, 1991).

In adults, Kit is expressed in many normal cell types, including bone marrow cells, germ cells, mast cells, and melanocytes (Lammie *et al.*, 1994), and the Kit:SCF interaction has been shown to stimulate proliferation (Metcalf and Nicola, 1991; Matsui *et al.*, 1991; Tsai *et al.*, 1991), maturation (Nocka *et al.*, 1990; Tsai *et al.*, 1991), migration and survival (Dolci *et al.*, 1991; Godin *et al.*, 1991) of these cells. Additionally, SCF activation of Kit has been shown to promote mast cell chemotaxis (Meininger *et al.*, 1992), adhesion to extracellular matrix components (Dastyk and Metcalfe, 1994; Kinashi and Springer, 1994), attachment to fibroblasts (Adachi *et al.*, 1992), and to prevent apoptosis (Iemura *et al.*, 1994; Yee *et al.*, 1994b; Mekori *et al.*, 1995; Ito *et al.*, 1999). Survival of intestinal pacemaker Cajal cells is dependent on the Kit:SCF interaction, and mutants have impaired autonomic gut motility (Maeda *et al.*, 1992; Ward *et al.*, 1995; Yamataka *et al.*, 1996). Additionally, Kit and SCF co-expression have been observed in the central nervous system and may be involved in neuronal organization, hippocampal learning and astrocyte survival (Morrison-

Graham and Takahashi, 1993; Motro *et al.*, 1996). Finally, the c-*KIT*:SCF system is also a key regulator of human testicular development and function, and defects in expression of this growth factor receptor-ligand pair have been implicated in developmental abnormalities, infertility and testicular cancer (Mauduit *et al.*, 1999).

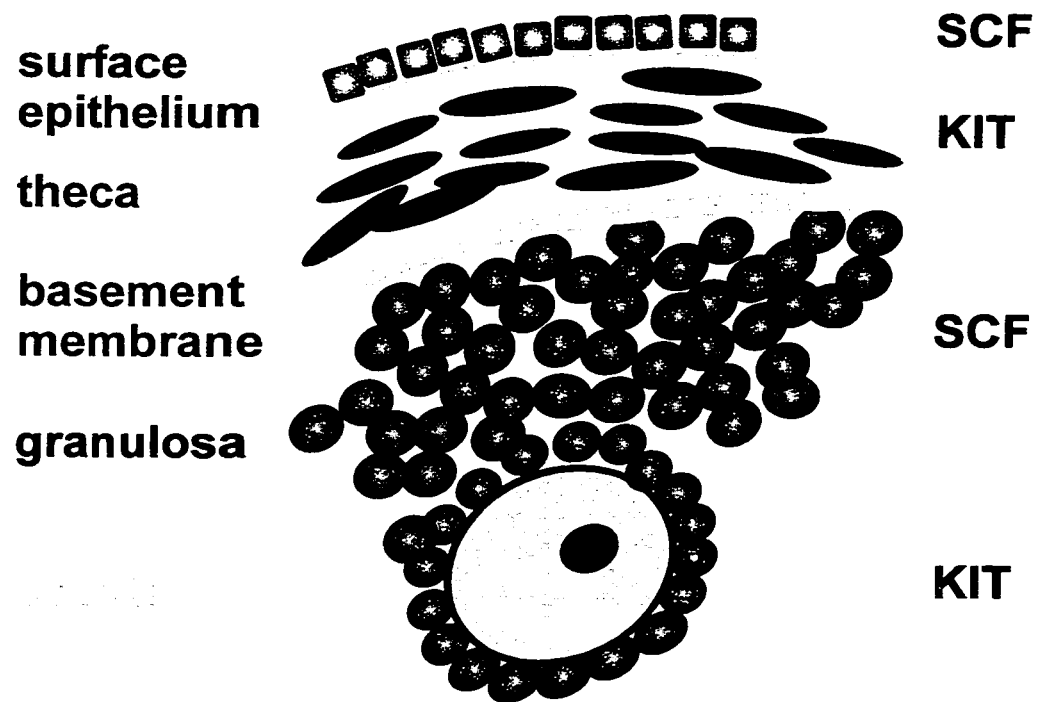
1.11 KIT and SCF Expression and Function in the Normal Ovary

Kit receptors have been detected on oocytes at all stages of follicular development in mouse (Manova *et al.*, 1990; Orr-Urtreger *et al.*, 1990; Horie *et al.*, 1991), rat (Ismail *et al.*, 1997), and human (Horie *et al.*, 1993; Tanikawa *et al.*, 1998), as well as on theca-interstitial cells of antral follicles of adult mice (Manova *et al.*, 1993; Motro and Bernstein, 1993). SCF is produced by granulosa cells in mice (Manova *et al.*, 1993; Motro and Bernstein, 1993), rats (Ismail *et al.*, 1996), humans (Laitinen *et al.*, 1995), and sheep (Tisdall *et al.*, 1997), as well as by rat OSE cells (Ismail *et al.*, 1999).

The alternate layering of Kit and SCF expression in the normal ovary (Figure 4) provides opportunities for functional paracrine interactions between ovarian cell types. Interaction of SCF produced by granulosa cells with Kit receptors on oocytes is required for oocyte growth (Packer *et al.*, 1994; Reynaud *et al.*, 2000) and normal follicular development (Kuroda *et al.*, 1988; Huang *et al.*, 1993; Bedell *et al.*, 1995; Yoshida *et al.*, 1997; Kissel *et al.*, 2000; Reynaud *et al.*, 2000) in postnatal animals. SCF activation of Kit on postnatal rat oocytes has been reported to inhibit meiotic progression of the oocytes (Ismail *et al.*, 1996; Ismail *et al.*, 1997). SCF can also stimulate the recruitment, proliferation and functional differentiation of bovine theca cells (Parrott and Skinner, 1997; Parrott and Skinner, 1998;

Figure 4

Expression of KIT and SCF in the ovary. Studies in mouse, rat, and human species have indicated that KIT and SCF are expressed in alternating layers of ovarian cells, with KIT being expressed by oocytes and theca-interstitial cells and SCF by granulosa cells and the surface epithelium. Picture courtesy of B. Vanderhyden.



Parrott and Skinner, 2000). In humans, the KIT:SCF interaction has been implicated in controlling the function of the ovarian follicle during the human menstrual cycle (Laitinen *et al.*, 1995).

1.12 Expression of KIT and SCF in Human Cancers

Expression of c-*KIT* mRNA and/or KIT protein has been reported in numerous human solid tumors and/or cancer cells, including bladder (Natali *et al.*, 1992a), breast (Natali *et al.*, 1992a; Natali *et al.*, 1992b; Hines *et al.*, 1995), cervix (Inoue *et al.*, 1994; Tsuura *et al.*, 1994), endometrium (Arber *et al.*, 1998), gastrointestinal stromal/smooth muscle (Ernst *et al.*, 1998), glioblastoma (Yarden *et al.*, 1987; Lerner *et al.*, 1991; Matsuda *et al.*, 1993), leukemia (Sekido *et al.*, 1993; Natali *et al.*, 1995; Valverde *et al.*, 1996), lung (Hibi *et al.*, 1991; Natali *et al.*, 1992a; Matsuda *et al.*, 1993; Rygaard *et al.*, 1993; Sekido *et al.*, 1993; Hida *et al.*, 1994; Tsuura *et al.*, 1994; Arber *et al.*, 1998), melanoma (Natali *et al.*, 1992a; Natali *et al.*, 1992c; Arber *et al.*, 1998), neuroblastoma (Cohen *et al.*, 1994), testis (Strohmeyer *et al.*, 1991; Natali *et al.*, 1992a; Tsuura *et al.*, 1994; Izquierdo *et al.*, 1995; Strohmeyer *et al.*, 1995; Arber *et al.*, 1998), and thyroid (Natali *et al.*, 1992a; Natali *et al.*, 1995; Arber *et al.*, 1998).

Additionally, co-expression of SCF with c-*KIT* has been detected in some of the cancers, such as breast (Hines *et al.*, 1995), cervix (Inoue *et al.*, 1994), lung (Hibi *et al.*, 1991; Rygaard *et al.*, 1993; Papadimitriou *et al.*, 1995; Pietsch *et al.*, 1998), melanoma (Papadimitriou *et al.*, 1995), neuroblastoma (Cohen *et al.*, 1994), and testis (Bokemeyer *et al.*, 1996).

Our laboratory's interest in the expression and function of the *c-kit*:SCF system in normal ovarian functions (section 1.11) was logically extended to abnormal ovarian functioning, namely ovarian cancer. In contrast to our observations in normal rat OSE, two groups have reported expression of *c-KIT* mRNA and/or KIT protein in a small number of human epithelial ovarian cancers (Inoue *et al.*, 1994; Arber *et al.*, 1998), and some of these tumors co-expressed SCF (Inoue *et al.*, 1994).

1.13 Function of the KIT:SCF Interaction in Cancer Cells

Co-expression of KIT and SCF in human cancers suggests the possibility of autocrine growth stimulation of cancer cells. Indeed, stimulation of proliferation or clonogenic growth of KIT-bearing cells in response to SCF has been demonstrated for a number of human cancer cell lines, including lung (Sekido *et al.*, 1993; Papadimitriou *et al.*, 1995; Krystal *et al.*, 1996), leukemia (Crosier *et al.*, 1993; Sekido *et al.*, 1993), glioblastoma (Berdel *et al.*, 1992), melanoma (Papadimitriou *et al.*, 1995), prostate (Savarese *et al.*, 1998), testis (Bokemeyer *et al.*, 1996), as well as acute myelogenous leukemia (AML) blasts from patients (Lauria *et al.*, 1995). Similarly, decreased proliferation in response to neutralizing anti-KIT antibodies has been reported for KIT-expressing neuroblastoma cell lines (Cohen *et al.*, 1994) and primary testicular tumor cells (Kondoh *et al.*, 1995). In addition to affecting proliferation, Sekido *et al.* (1993) found that KIT-bearing lung cancer and melanoma cells exhibited a chemotactic response to a positive gradient of SCF.

In addition to SCF-mediated activation of the KIT receptor leading to proliferation of cells, some mutations have been identified in KIT that result in SCF-independent

activation and growth stimulation. Point mutations in aspartic acid (Asp) 816 in the phosphotransferase domain of KIT leading to constitutive tyrosine phosphorylation and kinase activity, in the absence of SCF, have been identified in mouse, rat, and human mast cell leukemia cell lines (Furitsu *et al.*, 1993; Tsujimura *et al.*, 1994; Tsujimura *et al.*, 1995). The activating KIT Asp816 mutation has also been found in mastocytosis patients (Nagata *et al.*, 1995; Longley *et al.*, 1996) and testicular tumors (Tian *et al.*, 1999). Retroviral infection of the Asp816 mutant KIT into normal murine lymphoid Ba/F3 and myeloid FDC-P1 cells (Kitayama *et al.*, 1995), the murine IC-2 mast cell line (Hashimoto *et al.*, 1996; Piao and Bernstein, 1996), and hematopoietic progenitor cells (Kitayama *et al.*, 1996) resulted in SCF-independent activation and growth stimulation. In addition, Tsujimura *et al.* (1996) identified an in-frame, activating deletion of 21 bp at codons 573-579 of the Kit juxtamembrane domain in the murine FMA3 mastocytoma cell line that was also constitutively active when introduced into the IC-2 mast cell line (Tsujimura *et al.*, 1996) and the Ba/F3 murine lymphoid cell line (Nakahara *et al.*, 1998). Activating point mutations and/or deletions in the juxtamembrane domain of KIT have also been found in human gastrointestinal stromal tumors (Ernst *et al.*, 1998; Hirota *et al.*, 1998; Lasota *et al.*, 1999).

However, a dramatic decrease or loss of KIT expression has been reported for some tumor specimens and cell lines compared to their normal counterparts, including breast (Natali *et al.*, 1992a; Natali *et al.*, 1992b; Tsuura *et al.*, 1994), melanoma (Lassam and Bickford, 1992; Natali *et al.*, 1992a; Natali *et al.*, 1992c), skin (Tsuura *et al.*, 1994), testis (Strohmeyer *et al.*, 1991), and thyroid (Natali *et al.*, 1992a; Natali *et al.*, 1995). Highly invasive and metastatic breast cancer cells and melanoma cells have also been shown to lack

KIT expression (Natali *et al.*, 1992*a*; Natali *et al.*, 1992*b*; Matsuda *et al.*, 1993). These data suggested that KIT could be involved in *inhibition* of growth, and that this function is lost upon transformation of some cancer cells. Indeed, introduction of c-*KIT* into highly metastatic, KIT-negative melanoma cells markedly decreased the tumorigenic and metastatic capabilities of the cells (Huang *et al.*, 1996). Furthermore, the c-*KIT*-transfected melanoma cells underwent apoptosis upon exposure to SCF (Huang *et al.*, 1996). Thus, although the KIT:SCF interaction in normal cells consistently functions to stimulate proliferation and survival, in cancer cells both growth-promoting and growth-inhibiting actions of this receptor-ligand pair have been documented.

1.14 The Oncogenic Potential of KIT

In support of the growth-promoting role of KIT in normal and cancer cells, KIT has been shown to be oncogenic *in vitro* and *in vivo*. Experiments in which either a chimeric or a wildtype c-*KIT* molecule was expressed in NIH 3T3 fibroblasts or MCF-7 breast cancer cells resulted in increased cell proliferation and a transformed phenotype, as evidenced by colony formation in soft agar (Lev *et al.*, 1990; Hines *et al.*, 1995; Caruana *et al.*, 1998) and tumor development in nude mice (Caruana *et al.*, 1998). The enhanced proliferative rate of the KIT-expressing breast cancer cells could be inhibited by the addition of Kit-blocking antibodies (Hines *et al.*, 1995). Interestingly, Caruana *et al.* (1999) recently showed that the membrane-associated isoform of KIT had a superior transforming ability in NIH 3T3 cells compared to s-KIT. *In vivo*, Kondoh *et al.* (1995) found that the Kit:SCF interaction was essential for the development of testicular tumors in papillomavirus transgenic mice.

Additionally, expression of the activating Asp816 Kit mutants in normal Ba/F3 and FDC-P1 cells (Kitayama *et al.*, 1995) and murine IC-2 mast cells (Hashimoto *et al.*, 1996; Piao and Bernstein, 1996) resulted in cells that were tumorigenic in nude (Kitayama *et al.*, 1995; Hashimoto *et al.*, 1996) and syngeneic (Piao and Bernstein, 1996) mice. Indeed, transgenic mice expressing an Asp816 mutant Kit developed acute leukemia or malignant lymphoma (Kitayama *et al.*, 1996). Similarly, expression of the juxtamembrane point mutation or deletion Kit mutants in Ba/F3 cells (Hirota *et al.*, 1998; Nakahara *et al.*, 1998) and IC-2 mast cells (Tsujimura *et al.*, 1996) yielded cells that were tumorigenic in nude mice.

1.15 Regulation of KIT and SCF Expression in Normal and Cancer Cells

There are a number of growth factors, hormones, and second messengers which have been shown to regulate KIT and/or SCF expression in normal and cancer cells, often in conjunction with proliferative effects. TGF- β -induced growth suppression is mediated via inhibition of *c-kit* expression in murine and human hematopoietic progenitor cells (Dubois *et al.*, 1994; Heinrich *et al.*, 1995), AML blasts (de Vos *et al.*, 1993), and colorectal carcinoma cells (Bellone *et al.*, 1997). Downregulation of *c-KIT* mRNA and protein by exogenous TGF- β has also been associated with the maintenance of a quiescent state in human hematopoietic cells (Sansilvestri *et al.*, 1995). TGF- β has been shown to decrease SCF expression in human hematopoietic progenitor cells (Heinrich *et al.*, 1995), murine fibroblasts (Sugimoto *et al.*, 1999), and rat OSE cells (Ismail *et al.*, 1999). Additionally, low SCF in patients with sickle cell anemia has been associated with elevated TGF- β (Croizat and Nagel, 1999), and TGF- β inhibited the SCF-mediated rescue from apoptosis of IL-3-

deprived mast cells (Mekori and Metcalfe, 1994). Another TGF- β family member, growth differentiation factor-9 (GDF-9), has recently been shown to decrease expression of SCF in murine granulosa cells *in vivo* (Elvin *et al.*, 1999). Inhibition of SCF-induced proliferation of normal CD34⁺ hematopoietic cells by TNF- α was due to downregulation of *c-KIT* transcripts (Khoury *et al.*, 1994). Additionally, IL-1 upregulation of *c-kit* expression has been reported in murine bone marrow cells (Neta *et al.*, 1994) and human leukemia cells (Tomeczkowski *et al.*, 1998). Furthermore, a reduction of *c-kit* mRNA expression and surface receptor numbers occurred following addition of erythroid differentiation factor/activin A to murine erythroleukemia cells (Hino *et al.*, 1995).

Gonadal KIT and SCF expression can also be modulated by hormones. FSH and hCG induced significant increases in SCF mRNA transcripts in the granulosa cells of mice (Motro and Bernstein, 1993) and rats (Ismail *et al.*, 1996) *in vivo* and bovine granulosa cells *in vitro* (Parrott and Skinner, 1998), and primary cultures of mouse Sertoli cells were similarly affected by treatment with FSH (Rossi *et al.*, 1993). Conversely, FSH or hCG inhibited SCF mRNA expression in *in vitro* cultures of human granulosa-luteal cells (Laitinen *et al.*, 1995), suggesting that there may be species differences in the response of granulosa cells to gonadotropins. Expression of Kit receptors on murine theca-interstitial cells was decreased in response to LH (Motro and Bernstein, 1993), while oocytic Kit expression was unaffected by gonadotropins (Horie *et al.*, 1991; Motro and Bernstein, 1993; Ismail *et al.*, 1997).

Expression of the Kit-SCF pair can also be modulated by agents which elevate intracellular adenosine 3',5'-cyclic monophosphate (cAMP), the downstream second

messenger for FSH-R and LH-R. *C-kit* expression was increased in response to protein kinase A (PKA) activators like dibutyryl cAMP (dbcAMP) or forskolin in malignant hematopoietic cells (Ogawa *et al.*, 1995) and differentiated F9 teratocarcinoma cells (Nishina *et al.*, 1992), while *c-kit* expression was downregulated by the PKC activator 12-O-tetradecanoylphorbol-13-acetate in several cell types (Nishina *et al.*, 1992; Asano *et al.*, 1993; Ogawa *et al.*, 1995). DbcAMP also stimulated SCF mRNA accumulation in murine granulosa (Packer *et al.*, 1994) and Sertoli cells (Rossi *et al.*, 1993; Tajima *et al.*, 1993), as well as rat OSE cells (Ismail *et al.*, 1999). Thus, *c-kit* and SCF expression are differentially modulated by a number of factors in a number of different cell types.

1.16 Project Rationale

This Ph.D. project was undertaken, commencing in January 1996, based on a combined interest in the *c-KIT* protooncogene and ovarian cancer. Despite significant progress having been made in elucidating molecular genetic defects related to epithelial ovarian tumorigenesis, it was clear that more information was needed, particularly in identifying molecules responsible for malignant transformation of OSE cells that might be applicable as early biomarkers of the disease. Co-expression of the protooncogene *c-KIT* and its ligand, SCF, had been demonstrated in a number of solid tumors, and stimulation of proliferation in response to SCF had been reported for several types of cancer cells. An oncogenic potential of activated KIT had also been demonstrated *in vitro* and *in vivo*. Furthermore, there was a single report of co-expression of KIT and SCF in a small number of epithelial ovarian tumors (Inoue *et al.*, 1994), which suggested the possibility of autocrine

growth regulation in ovarian cancer cells. Lastly, preliminary results in our laboratory (now published) had shown that primary rat OSE cells did not express *c-kit* but did express SCF (Ismail *et al.*, 1999). Thus, **we hypothesized that, if the same held true for human OSE, aberrant expression of c-KIT in OSE cells, which normally express SCF, would allow for an autocrine growth regulatory loop in the OSE and OSE-derived tumors which could contribute to the initiation and/or progression of human epithelial ovarian cancer.** The specific objectives of this study were, therefore, as follows:

- (1) To determine whether normal human OSE cells express *c-KIT* and/or SCF;
- (2) To determine the frequency of KIT and SCF expression in epithelial ovarian cancers;
- (3) To determine the cellular localization of KIT in the epithelial ovarian tumors;
- (4) To correlate the expression of *c-KIT* with tumor grade and stage, as well as with patient survival;
- (5) To identify factors which regulate *c-KIT* and SCF expression in ovarian cancer cells *in vitro*; and
- (6) To elucidate a growth regulatory function of the KIT:SCF interaction in ovarian cancer cells *in vitro*.

Chapter 2 - General Materials and Methods

A number of experimental methodologies were common among studies described in Chapters 3, 4 & 5 and, therefore, a description of those methodologies is provided here. Any studies that contain experimental details that differ from the general methods or that are unique to that chapter are described separately within that chapter.

2.1 Cell Lines

A variety of cell lines were used in numerous applications in this research project, and Table 1 provides detailed information regarding these lines. For routine maintenance, the cells were passaged every 3-5 days, generally at 95% confluence. Passaging entailed aspirating the spent medium, washing the cells with phosphate-buffered saline (PBS; 8 g/l NaCl, 0.2 g/l KCl, 0.2 g/l KH_2PO_4 , 1.15 g/l Na_2HPO_4), and trypsinizing the cells from the dish by incubation at 37°C in the presence of 0.025% trypsin and 1 mM EDTA in PBS. The cells were pelleted by centrifugation at 3,000 rpm for 3 min, resuspended in fresh media, and seeded into new dishes. All of the cell lines were grown in monolayer culture with the exception of the murine mast cell line, MC/9, which was grown in suspension culture. The cells were all cultured at 37°C in 5% CO_2 and 95% humidified air. Specific experimental usage details are given within the subsequent chapters.

Table 1. Cell lines summary¹.

CELL LINE (received from)	SOURCE	ORIGINAL REFERENCE	CULTURE MEDIUM
A2780-s (M. Molepo)	untreated ovarian cancer patient	Eva <i>et al.</i> , 1982	D-MEM/F12 + 0.84 g/l NaHCO ₃ + 4.77 g/l HEPES + 10 ml/l NEAA + 7.5% NCS + 7.5% FCS
A2780-cp (M. Molepo)	stepwise exposure of A2780-s cells to up to 70 µM cisplatin	Masuda <i>et al.</i> , 1988	D-MEM/F12 + 0.84 g/l NaHCO ₃ + 4.77 g/l HEPES + 10 ml/l NEAA + 7.5% NCS + 7.5% FCS
LC-73 (D. Gray)	squamous cell lung cancer	Gray <i>et al.</i> , 1995	α-MEM + 10% FCS
ACC-LC-80 (D. Gray)	small cell lung cancer	Hibi <i>et al.</i> , 1991	α-MEM + 10% FCS
C13* (M. Molepo)	stepwise exposure of OV2008 cells to 0.25-5.25 µM cisplatin	Andrews and Albright, 1992	RPMI + 10% FCS
HEY (G. Mills)	serous Ad, solid tumor	Buick <i>et al.</i> , 1985	α-MEM + 10% FCS
MC/9 (ATCC)	mast cell clone derived from fetal liver cells of (B6XA/J)F ₁ mouse	Galli <i>et al.</i> , 1982	45% modified D-MEM + 45% splenocyte-conditioned media + 10% FCS
NIH 3T3 (D. Gray)	NIH Swiss mouse embryo fibroblast culture	Jainchill <i>et al.</i> , 1969	α-MEM + 10% FCS
OCC1 (G. Mills)	serous Ad, solid tumor	Wong <i>et al.</i> , 1990	α-MEM + 10% FCS
OCIM-1 (H. Atkins)	erythroleukemia	Papayannopoulou <i>et al.</i> , 1984	IMDM + 5% FCS
OV2008 (M. Molepo)	serous Ad, solid tumor, stage IV	DiSaia <i>et al.</i> , 1972	RPMI + 10% FCS
OVCA 420 (G. Mills)	Ad, ascites	Berchuck <i>et al.</i> , 1992	α-MEM + 1% NEAA + 10% FCS
OVCA 429 (G. Mills)	Ad, ascites	Berchuck <i>et al.</i> , 1992	α-MEM + 1% NEAA + 10% FCS
OVCA 432 (G. Mills)	Ad, ascites	Berchuck <i>et al.</i> , 1992	α-MEM + 1% NEAA + 10% FCS
OVCA 433 (G. Mills)	Ad, ascites	Berchuck <i>et al.</i> , 1992	α-MEM + 1% NEAA + 10% FCS
OVCAR-3 (G. Mills)	Ad, ascites	Hamilton <i>et al.</i> , 1983b	α-MEM + 10% FCS
ROSE 199 (N. Auersperg)	rat OSE, spontaneously immortalized	Adams and Auersperg, 1985	α-MEM + 10% FCS
SKOV-3 (G. Mills)	Ad, ascites	Fogh and Trempe, 1975	McCoy's 5A + 2.2 g/l NaHCO ₃ + 15% FCS

¹ For an explanation of abbreviations please refer to the List of Abbreviations and Chemical Formulae.

2.2 Proliferation

Several studies assessed cellular proliferation as an experimental endpoint. Proliferation was measured either by counting the cells using hemacytometers (VWR Scientific) or by determining the amount of [methyl-³H]thymidine (Amersham, Oakville, ON; hereafter referred to as [³H]thymidine) that was incorporated into DNA during a particular time frame.

2.2.1 Hemacytometry

For hemacytometer counting of cells, 10 µl of the cell suspension was applied to each of the 2 counting grids of the hemacytometer, and the average of the 2 cell counts was used in determining the mean for a treatment group, i.e., since each treatment group was usually done in triplicate, a mean of 3 replicates was determined from a total of 6 separate counts. A minimum of 20 cells per square of the hemacytometer grid was considered to be the fewest number of cells that could accurately reflect the total number of cells in the suspension.

In some studies where decreased cell proliferation was observed, determination of cell viability was done by trypan blue dye exclusion. The trypan blue exclusion test as a measure of viability is based on the premise that viable cells are normally impermeable to trypan blue (Kaltenbach *et al.*, 1958), but that dying or dead cells likely have compromised plasma membranes so will be unable to exclude the dye and will appear blue when viewed under the microscope. Thus, for determination of cell viability the cells were resuspended for hemacytometer counting in media containing 0.03% trypan blue solution (Gibco, Grand Island, NY), and white (viable) cells versus blue (non-viable) cells were counted. Percent

viability was determined by calculating: (number of white cells/number of white cells + number of blue cells) x 100%.

2.2.2 [³H]Thymidine incorporation

For determination of [³H]thymidine incorporation into cellular DNA as a measure of proliferation, cells grown in monolayer culture in 24-well plates were exposed to 1.0 µCi/ml of [³H]thymidine during the last 6-20 h of the experimental culture time. The radioactive media was removed and the cells were incubated with 1 ml/well of cold (i.e., non-radioactive) thymidine solution (100 µg/ml in culture medium) at room temperature for 15 min in order to displace any [³H]thymidine that may be bound to the outside surface of the cells. The thymidine solution was removed and the cells were gently washed, twice, using 500 µl/well of Dulbecco's PBS (D-PBS; 0.133 g/l CaCl₂, 0.1 g/l MgCl₂, 0.2 g/l KCl, 0.2 g/l KH₂PO₄, 8 g/l NaCl, 1.15 g/l Na₂HPO₄, pH 7.4). The washed cells were then incubated for 10 min at room temperature in the presence of 500 µl/well of 0.5% SDS solution (in D-PBS) to lyse the cells. Each lysed cell suspension was transferred to a scintillation vial, then an additional 500 µl of the 0.5% SDS solution was used to rinse each well, and the rinsate was added to the corresponding scintillation vial. Five ml of scintillation fluid (Amersham) was added to each vial, and the amount of tritium radioactivity [in counts per minute (cpm)] was counted using a Packard 1600 TR Liquid Scintillation Analyzer (Packard Instrument Co., Meriden, CT). Values were expressed as a percent of the experimental control group, which was arbitrarily set to 100%. Background controls were included in each assay: vials containing only the scintillation fluid, and vials that contained 1 ml of the 0.5% SDS lysis

solution plus the scintillation fluid were counted as an indication of the background cpm. Note that no correction for background cpm was made to the experimental values reported herein since the average background count from all of the experiments was only 13 cpm (range 4-52) and was considered to be insignificant when compared to the experimental values which were in the thousands of cpm.

2.3 Northern Blot Analysis

2.3.1 Extraction and quantification of RNA

Total RNA was extracted from cultured cells using a Qiagen RNeasy Mini Kit (Qiagen Inc., Mississauga, ON) following the manufacturer's instructions. For extraction of total RNA from epithelial ovarian tumors, a small piece of frozen tumor was pulverized using a mortar and pestle on dry ice. The tumor fragments were immediately dropped into a 50 ml Falcon tube (on ice) containing 5 ml of ice-cold 6 M urea/3 M LiCl solution. The tumor sample was homogenized for 2.5 min, and then incubated at 4°C overnight to precipitate the RNA. The tumor suspension was centrifuged at 9,000 rpm for 30 min at 0°C, and the pellet was resuspended in 2.5 ml of 3 M LiCl using a 1 ml Sub-Q 26G syringe (Becton Dickinson), then again centrifuged at 9,000 rpm for 30 min at 0°C. The pellet was resuspended in 500 µl of a solution containing 10 mM Tris-HCl pH 7.5, 0.5% SDS, 50 µg/ml proteinase K (Boehringer Mannheim) and incubated at 37°C for 20 min. The tumor RNA was then extracted using an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) twice and chloroform:isoamyl alcohol once, pelleted, and resuspended in 100-200 µl of RNase-free ddH₂O. RNA concentration was determined by measuring the absorbance (abs)

at $\lambda = 260/280$ nm on a Beckman DU® 640 Spectrophotometer (Beckman Instruments Inc., Mississauga, ON) that was blanked with RNase-free ddH₂O prior to reading the samples. An abs of 1.0 at $\lambda = 260$ nm reflected a concentration of 40 $\mu\text{g/ml}$ of total RNA in the sample.

2.3.2 Gel electrophoresis and hybridization

Total cellular or tumor RNA (15 μg unless otherwise indicated) was separated in denaturing 0.9% agarose gels containing 5% (v/v) formaldehyde. The RNA was transferred by capillary action overnight to Hybond N nylon membranes (Amersham) and cross-linked on the membranes at 100 mJ in a GS Gene Linker UV Chamber (Bio-Rad Laboratories, Hercules, CA). The blots were hybridized using a standard protocol (Sambrook *et al.*, 1989) with [³²P]cDNA probes labeled by random priming (Boehringer Mannheim). The blots were probed consecutively with a human *c-KIT* cDNA (5100 bp), a human SCF cDNA (920 bp), and a tubulin cDNA, with each blot stripped once using a 0.1% boiling SDS solution following the *c-KIT* probing. The plasmids containing human *c-KIT* and SCF were generously donated by Dr. F. Jacobsen (Amgen Inc., Thousand Oaks, CA), and the tubulin cDNA was kindly provided by Dr. M. McBurney (Ottawa, ON). Post-hybridization washing of the blots was done with a 2XSSC, 0.1% SDS solution for 2x15 min, with one change of solution, followed by stringent washing at 65°C with either 0.5XSSC, 0.1% SDS for 2x15 min (*c-KIT* and SCF probes) or 0.1XSSC, 0.1% SDS for 2x30 min (tubulin probe), with one change of solution in both cases. Washed blots were exposed to phosphor screens, scanned using a PhosphorImager SI (version 4.0, Molecular Dynamics, Sunnyvale, CA), and analyzed using ImageQuaNT software (version 4.2a, Molecular Dynamics).

2.4 Reverse Transcription-Polymerase Chain Reaction

Reverse transcription-polymerase chain reaction (RT-PCR) analysis was done on total RNA isolated from cells and ovarian tumors to determine the relative expression of membrane-associated and soluble isoforms of *c-KIT* and SCF.

Primers for *c-KIT* were designed with Oligo5[®] software to span at least one intron and the 12 bp region which differentiates the transcripts encoding the membrane-associated and soluble isoforms of the KIT receptor (Yarden *et al.*, 1987). Reverse transcription of 1.5 µg of RNA was carried out with 200 U of Moloney murine leukemia virus reverse transcriptase (Superscript[™] RNase H⁻, Gibco; unless otherwise indicated, all RT-PCR reagents were obtained from Gibco) with 0.005 µg of oligo(dt)₁₂₋₁₈ primer (Boehringer Mannheim) in a 20 µl reaction mixture consisting of 10 U RNase inhibitor, 1X 1st strand buffer (50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM MgCl₂), 0.01 M DTT and 0.625 mM each of dATP, dCTP, dGTP and dTTP (Boehringer Mannheim). Primer annealing was carried out at 70°C for 5 min in the presence of RNase inhibitor. First strand synthesis followed at 42°C for 60 min with the addition of 1st strand buffer, DTT, dNTPs and Superscript. The reverse transcriptase was then denatured at 94°C for 5 min and the cDNA was stored at -20°C. Five µl of the cDNA was amplified in each PCR reaction with 0.02 U *Taq* DNA polymerase in a 50 µl reaction mixture consisting of 1X *Taq* PCR buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl), 3 mM MgCl₂ and 0.5 pmol each of the 5' primer (5'-CCC AGA GCC CAC AAT AGA TTG-3') and 3' primer (5'-CGT TCT GTC AAA TGG GCA CTC-3'). The nucleotide positions of the 5' and 3' *c-KIT* primers were bp 1320-1341 and bp 1902-1922, respectively, of the GenBank accession no. X06182. The PCR amplification was carried out in a Perkin Elmer

GeneAmp® PCR System 2400 thermal cycler with the following cycling profile: 94°C for 5 min; 68°C for 2 min; 38 cycles of 94°C and 68°C for 1 and 2 min, respectively, followed by 94°C for 1 min; and 72°C for 10 min. The reaction was held at 4°C until storing at -20°C.

In order to clearly distinguish between the fragments representative of the membrane-associated and soluble isoforms (591 bp and 603 bp, respectively) of the *c-KIT* receptor, separation of the amplified products was done on a 4% agarose gel in 1XTAE buffer (0.04 M Tris-acetate/0.001 M EDTA) overnight at 4°C. Additionally, the identity of the *c-KIT* fragments was confirmed by using digestion at a unique restriction site, present only in the transcript encoding the soluble isoform, and automated sequencing. Forty percent of the PCR reaction was digested at 55°C with 1.5 U *Mae III* enzyme (Boehringer Mannheim) in a 50 µl reaction using 1X *Mae III* digestion buffer (20 mM Tris-HCl, 275 mM NaCl, 6 mM MgCl₂, 7 mM 2-mercaptoethanol, pH 8.2 at 55°C). The digest was then electrophoretically separated on a 2% agarose gel. Fragments running at the expected sizes of 229 bp and 374 bp were excised, purified (Geneclean kit, BIO 101 Inc., La Jolla, CA), and sequenced with a Perkin Elmer automated sequencer.

RT-PCR analysis to examine the relative expression of membrane-associated and soluble SCF was performed as described for the RT-PCR analysis of *c-KIT* isoforms with the following differences. The SCF primers spanned the alternatively-spliced exon 6, thereby enabling amplification of the 287 bp and 203 bp fragments representative of the soluble and membrane-associated SCF isoforms, respectively, as previously reported (Ismail *et al.*, 1997). The RT reaction was done in 1X *Taq* buffer with 1.25 mM of each dNTP and 4 mM MgCl₂, without DTT. First strand synthesis was carried out for 1 h at 37°C. The PCR

reaction mixture contained 25 pmol of each SCF primer [5'primer (5'-GTA TTT TCA ATA GAT CCA TTG A-3'), 3'primer (5'-CCA GTA TAA GGC TCC AAA AGC AA-3'); Gibco] with 1 mM MgCl₂. The nucleotide positions of the 5' and 3' SCF primers were bp 530-551 and bp 710-732, respectively, of the GenBank accession no. S40534. A Perkin Elmer 9600 thermal cycler was used to carry out the following cycling profile: 3 cycles of 94°C, 60°C and 72°C for 5, 2 and 3 min, respectively; 30 cycles of 94°C, 60°C and 72°C for 1, 2 and 3 min, respectively, followed by a final extension at 72°C for 10 min.

All c-*KIT* and SCF PCR gels were stained in SYBR green (Molecular Probes Inc., Eugene, OR) at the recommended dilution of 1:10,000 in water, and visualized on a STORM 860 Phosphor/Fluorescence Imager (Molecular Dynamics) using the blue fluorescence mode.

2.5 Western blot analysis

Total protein lysates were obtained from cultured cells or ovarian tumors using a standard protocol (Sambrook *et al.*, 1989). The concentration of protein in the lysates was quantified with a commercially available assay (Bio-Rad Laboratories) using BSA (ICN Biomedicals Inc.) as the standard. Denatured protein samples (60 µg of cellular lysates or up to 425 µg of tumor lysates) and protein markers (New England BioLabs, Beverly, MA) were separated on 5% polyacrylamide gels and electroblotted overnight to Hybond C Extra nitrocellulose membranes (Amersham, Oakville, ON) in a solution containing 0.2 M glycine, 0.02 M Tris-HCl, and 20% (v/v) methanol. The blots were blocked in a 10 mM Tris-HCl, 180 mM NaCl, 0.05% (v/v) Tween-20 (TBST) solution containing 5% (w/v) skimmed milk powder for 1 h with shaking. Blots were washed 5 min with TBST and then incubated with

a mouse anti-human KIT MAb (Boehringer Mannheim; 0.5 µg/ml in TBST containing 20% FCS) for 1 h with shaking. After washing for 3x5 min in TBST, blots were incubated with a goat anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody (Bio-Rad Laboratories; diluted 1:5000 in primary antibody diluent) for 45 min with shaking. Following 3x5 min washes with TBST, immunoreactive bands were visualized using the LumiGLO Chemiluminescent Substrate Kit (Kirkegaard & Perry Laboratories, Gaithersburg, MD) and Kodak X-Omat Blue XB-1 film (Eastman Kodak Company, Rochester, NY).

In some experiments, KIT protein was immunoprecipitated from cells prior to western blot analysis. Following culture, cells were rinsed twice with cold PBS and then placed on ice. To each 100 mm dish was added 200 µl of Frack's buffer [20 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 2 mM NaF, 2 mM NaPP, 500 µM (NH₄)₃VO₄, 200 µg/ml PMSF, 2 µg/ml aprotinin, and 2 µg/ml leupeptin]. The cells were lysed for 20 min on ice, then the lysates were scraped from the dishes into eppendorf tubes using a rubber policeman. The lysates were vortexed for 10 sec, and the cellular debris was pelleted at 14,000 rpm for 10 min at 4°C. The total amount of protein in each supernatant was quantified as described above. A 1.5 mg quantity of each protein lysate was used for the immunoprecipitation.

GammaBind[®]G Sepharose[®] beads (Pharmacia Biotech, Baie d'Urfé, PQ) were prepared by twice washing 100 µl of the bead slurry with 1 ml of Frack's buffer, and then resuspending the beads 1:1 in Frack's buffer. The lysates were pre-cleared by incubating in the presence of 50 µl of the slurry on a rotating shaker at 4°C overnight. After pelleting the beads by brief centrifugation, 5 µg of an anti-KIT MAb (Boehringer Mannheim) was added

to each supernatant and incubated for 1.5 h on a rotating shaker at 4°C. To immunoprecipitate the KIT:anti-KIT antibody complexes, the lysates were then incubated with 50 µl of bead slurry for 1 h with shaking at 4°C. The beads with the bound complexes were washed 4 times with 1 ml of Frack's buffer, then the beads were resuspended in 25 µl of 2x Sample Buffer (0.125 M Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 5% 2-mercaptoethanol, 0.05% bromophenol blue). Western blot analysis was carried out as described above.

2.6 Immunocytochemistry

2.6.1 Immunolocalization of keratin

Confirmation of the epithelial phenotype of primary human OSE cells was done by immunolocalization of epithelial keratin in cells cultured on 0.15% gelatin-coated coverslips. Cells were fixed in -20°C methanol for 5 min, air-dried for 5 min and rehydrated with Stockholm PBS (S-PBS; 4.6 g/l Na₂HPO₄, 1.05 g/l NaH₂PO₄, 32 g/l NaCl, 0.8 g/l KCl) for 15 min. The cells were then incubated with an anti-keratin AE1/AE3 pooled MAb (Boehringer Mannheim; 20:1 mixture of AE1 to AE3; diluted 1:50 in S-PBS containing 1% BSA, 0.1% sodium azide, and 0.2% Triton X-100) in a humidified chamber for 1 h. The anti-keratin AE1 antibody recognizes the 40, 48, 50, 50' and 56.5 kDa keratins of the acidic subfamily, while the AE3 antibody recognizes all of the basic keratins. Following 3x5 min washes in S-PBS, the cells were incubated with a biotinylated anti-mouse IgG secondary antibody (Amersham; diluted 1:200 in S-PBS) for 1 h. After 3x5 min washes with S-PBS, cells were incubated with fluorescein isothiocyanate (FITC)-conjugated streptavidin

(Amersham; diluted 1:200 in S-PBS) for 30 min. Following 3x5 min washes with S-PBS, coverslipped cells were mounted onto slides using 10 μ l of anti-fade mounting medium (1 mg/ml p-phenylenediamine, 10% glycerol in 0.9% NaCl). Cells were viewed on a Zeiss Axiophot inverted microscope (Carl Zeiss Canada Ltd., Don Mills, ON) equipped with epifluorescent optics. Digital images were obtained using a CCD Video Camera System (Carl Zeiss Canada Ltd.) and Intellicam software (version 2.0; Matrox, Dorval, PQ).

2.6.2 Immunolocalization of KIT

Identification of KIT-expressing cells and the subcellular localization of KIT protein was carried out as detailed for the immunocytochemical localization of keratin in normal human OSE cells (section 2.6.1), but using different pairs of antibodies. To localize human KIT, a primary rabbit anti-KIT polyclonal antibody (Oncogene, Cambridge, MA) targetted against amino acid residues 961-976 at the carboxy-terminal end of KIT (diluted 1:10 in S-PBS/0.05% Triton X-100) was used with a CYTM3-conjugated donkey anti-rabbit secondary antibody (diluted 1:200 in S-PBS; Jackson ImmunoResearch Laboratories Inc., West Grove, PA). To identify murine Kit protein, a primary rat anti-Kit MAb, ACK2 (diluted 1:500 in S-PBS, 1% BSA, 0.1% sodium azide, 0.2% Triton X-100; kindly provided by Dr. S.-I. Nishikawa, Kumamoto, Japan), targetted against the extracellular domain of KIT, was used in conjunction with an FITC-conjugated rabbit anti-rat IgG (diluted 1:50 in S-PBS; Dako Corp., Denmark). In some experiments, cells were fixed with Lana's fix [4% paraformaldehyde, 14% saturated picric acid (v/v) in 0.25 M NaH₂PO₄, pH 6.9] for 15 min at RT instead of methanol.

2.7 Statistical Analyses

All cell counts were expressed as the mean $\pm$ standard error of the mean (SEM) of at least 6 different values derived from a minimum of 3 separate experiments. The probability of significant differences among multiple treatment groups was determined by analysis of variance (ANOVA). Post-tests (Bonferroni's, Fisher's Protected Least Squares Differences, or Newman-Keuls Multiple Comparison Test, depending on the software program) were used to determine significance between specific treatment groups when whole group differences were detected by ANOVA. For all analyses, significance was inferred at $p < 0.05$.

Chapter 3 - Lack of Expression of c-*KIT* in Ovarian Cancers is Associated with Poor Prognosis

3.1 Introduction

Ovarian cancer is the most common gynecologic malignancy causing death in North America. The disease affects 1 in 70 women and its insidious growth pattern, combined with few specific early symptoms, commonly results in diagnosis of advanced stage disease upon clinical presentation. There is no reliable diagnostic screening method available, and the only risk factors to be clearly recognized to date are uninterrupted ovulation (Fathalla, 1971) and inherited mutations in the *BRCA1* or *BRCA2* genes (Takahashi *et al.*, 1995; Lancaster *et al.*, 1996). Over-expression and/or activation of several genes, including *HER-2/neu*, *myc*, *ras* and mutant *p53* has been described in some ovarian cancers (reviewed in Auersperg *et al.*, 1998). Co-expression of protooncogene receptors such as the EGF-R (Kohler *et al.*, 1989; Berchuck *et al.*, 1991) with their respective ligands has also been reported. Despite these observations, knowledge of the growth regulation and malignant transformation of human OSE, the cellular origin of almost 90% of ovarian tumors (Weiss *et al.*, 1977), remains incomplete.

Members of the tyrosine kinase class of growth factor receptors are key components of the biological control networks for cellular proliferation, differentiation and metastasis. Over-expression or mutation of these receptors, or the intracellular components of their signalling pathways, can disrupt growth regulation and result in tumor formation (Cross and Dexter, 1991). The protooncogene *c-KIT* encodes a tyrosine kinase receptor related to the

PDGF/CSF-1 receptor subfamily (Qiu *et al.*, 1988; Figure 3). KIT is activated by its ligand, SCF, and this interaction plays an important role in the development of several embryonic processes, including gametogenesis, melanogenesis and hematopoiesis (reviewed in Morrison-Graham and Takahashi, 1993), as well as a number of cell-specific functions in some adult tissues (Maeda *et al.*, 1992). As detailed in Chapter 1, the interaction of SCF with KIT also regulates the growth of many cancer cell types, and the oncogenic potential of KIT has been demonstrated both *in vitro* and *in vivo*.

Several studies have investigated the expression of c-*KIT* and SCF in epithelial ovarian cancers (Inoue *et al.*, 1994; Wrigley *et al.*, 1996; Arber *et al.*, 1998) but have yielded conflicting results. Despite the fact that all groups evaluated the tumors using immunohistochemistry, the percentage of tumors that expressed c-*KIT* ranged from 0 (n=46; Wrigley *et al.*, 1996) to 87% (n= 23; Arber *et al.*, 1998). Neither the function of KIT in these cancers nor the prognostic significance of its expression was determined. The objectives of this study, therefore, were to examine the expression of c-*KIT* and SCF in normal human ovaries, in cultured human OSE cells, and in human epithelial ovarian tumors, and to correlate these results with tumor pathology and patient survival.

3.2 Materials and Methods

3.2.1 Collection and culture of normal human OSE cells

Normal human OSE cells were collected from a single ovary of 5 different women following surgery for reasons other than ovarian pathology. OSE were obtained by firm

scraping of the ovarian surface with a scalpel blade. Cells were removed from the blade by immersion in 5 or 10 ml DMEM/F12 medium (Gibco) in a 50 ml Falcon tube (Becton Dickinson, Franklin Lakes, NJ) and were pelleted at 3,000 rpm. Following one wash with media, cells were resuspended by gentle pipetting with a large bore tip in medium containing 5 ng/ml EGF (Boehringer Mannheim, Laval, Canada), 5 µg/ml insulin (Sigma-Aldrich, Oakville, Canada), 10 µg/ml transferrin (Sigma), 5×10^{-5} M *O*-phosphoryl-ethanolamine (Sigma), 5×10^{-5} M ethanolamine (BDH, Toronto, Canada), 0.05 mg/ml gentamicin (Gibco) and 3% heat-inactivated fetal calf serum (FCS; CanSera, Rexdale, Canada). Cells were then plated into 60 mm Nunclon tissue culture dishes (Gibco) and cultured at 37°C in 5% CO₂ and 95% humidified air. Normal human OSE cells were allowed to attach over a period of 5 to 7 days, after which they were fed twice weekly with complete medium. Cell passage was carried out as described in section 2.1 when cultures reached a confluency of approximately 60-70%.

The epithelial phenotype of primary human OSE cells was confirmed morphologically and by immunolocalization of epithelial keratin in cells as described in section 2.6.1.

3.2.2 Culture of tumor cell lines

A number of tumor cell lines were used as positive and negative controls for KIT and SCF expression in protein and RNA analyses of normal human OSE cells and epithelial ovarian tumors. The cell lines used were ACC-LC-80, OCIM1, OVCAR-3, OVCA 420, OVCA 433, and ROSE 199 cells (Table 1), and the cells were cultured as described in

section 2.1.

3.2.3 Collection and storage of epithelial ovarian tumor specimens

The collection, storage and use of human epithelial ovarian tumors was approved by the Research Ethics Board of the Ottawa Hospital (General Site), and all patients had provided informed consent for the use of their tumor tissue for this study prior to surgery. All of the tumor specimens were obtained from patients undergoing their first surgery and who had not received any form of therapy. Following surgical excision from the patient, a 1-3 cm³ piece of tumor was cut (juxtapositional to the tissue slice used for histopathological frozen section diagnosis) with a sterile razor blade and immediately flash-frozen in liquid nitrogen. The frozen tumor specimen was subsequently wrapped in aluminum foil on dry ice, and stored in a 50 ml Falcon tube at -80°C. Tumors were staged at the time of surgery according to the FIGO staging system and are reported herein as stages I, II, III or IV with no further subdivision. Histologic grade of the tumors was assigned based on the permanent section morphology. Grade 1 refers to a well differentiated tumor, grade 2 indicates a moderately differentiated tumor and grade 3 refers to a poorly differentiated tumor.

3.2.4 Evaluation of *c-KIT* and SCF expression

Expression of *c-KIT* and SCF mRNAs in normal human OSE cells and epithelial ovarian tumors was determined by northern blot analysis as described in section 2.3. RT-PCR analysis to determine the relative expression of membrane-associated and soluble isoforms of *c-KIT* and SCF in primary human OSE cells and epithelial ovarian tumors was

done as described in section 2.4. Determination of the expression of KIT proteins in epithelial ovarian tumors was carried out as detailed in section 2.5.

3.2.5 Immunohistochemistry

KIT protein was immunolocalized in formaldehyde-fixed, paraffin-embedded 5 μ m sections of normal human ovaries and human epithelial ovarian tumors as follows. Sections were deparaffinized in 4 changes of toluene (5 min each), rehydrated in 2 changes of 100% ethanol (1 min each), and rinsed in running tap water followed by distilled water. After 2x5 min washes in TBS (0.05 M Tris-HCl, 0.15 M NaCl, pH 7.6), the sections were incubated in hydrogen peroxide (3% H₂O₂ in TBS) for 20 min to block endogenous peroxidase activity. The sections were washed 3x5 min in TBS, and then incubated with a general suppressor [4% BSA (Sigma), 10% sucrose, 1% FBS (Immunocorp, Montreal, Canada) in TBS, pH 7.6] for 30 min in a covered humidity chamber to block non-specific binding sites. Following aspiration of the suppressor, the sections were incubated with a polyclonal anti-KIT primary antibody (Oncogene; diluted 1:100 in 10% sucrose, 1% BSA, 0.01% sodium azide in TBS) for 24 h at 4°C in a covered humidity chamber. Sections on which the primary antibody was omitted were included as controls for the specificity of any observed staining. The sections were brought to room temperature, rinsed and washed 3x5 min in TBS, and then incubated with a peroxidase-labelled polymer secondary antibody (conjugated to anti-mouse and -rabbit immunoglobulins; DAKO Corporation, Carpinteria, CA) for 30 min in a covered humidity chamber. After 3x5 min washes in TBS, the sections were incubated in DAB chromogenic substrate solution [0.02% diaminobenzidine (Sigma), 0.001% H₂O₂ in TBS] for 10 min in

the dark with stirring, followed by immediate rinsing in TBS then running tap water. The sections were counterstained with hematoxylin (Shandon, Pittsburgh, PA), dehydrated in 100% ethanol, cleared in toluene and mounted using Permount (Fisher, McLean, VA). Digital images were obtained using a CCD Video Camera System (Zeiss, Don Mills, Canada) and Intellicam software (version 2.0; Matrox, Dorval, Canada).

3.2.6 Survival curves and statistical analyses

Kaplan-Meier survival plots of patients whose malignant ovarian tumors did and did not express *c-KIT* were plotted and statistically compared using a logrank test with GraphPad Prism software (version 2.0; GraphPad, San Diego, CA). Statistical significance was inferred at $p < 0.05$.

3.3 Results

3.3.1 Lack of *c-KIT* expression in normal human OSE

Normal human OSE cells grew in primary culture generally with a characteristic cobblestone appearance, but with epithelio-mesenchymal conversion as evidenced by a fibroblast-like morphology of some cells. Immunocytochemical detection of keratin in these OSE cells confirmed their epithelial phenotype (Figure 5). The cultured normal OSE cells consistently did not express *c-KIT* mRNA (5 of 5 independent primary OSE cell cultures), as assessed by northern blot analysis (Figure 6A) and by RT-PCR analysis (Figure 7). All primary OSE cells did, however, express SCF mRNA (6 kb transcript, Figure 6B), and

Figure 5. Expression of epithelial keratin in normal human ovarian surface epithelial cells. Methanol-fixed primary human OSE cells on coverslips were incubated with an anti-keratin AE1/AE3 pooled monoclonal antibody (recognizes both acidic and basic cytokeratins), followed by a biotinylated secondary antibody and an FITC-conjugated streptavidin tertiary antibody. Panels *a* and *c* are phase-contrast images of the immunofluorescent pictures in panels *b* and *c*. Normal OSE cells showed clear staining of cytoplasmic keratin filaments (*b*). No staining was observed if the primary antibody was omitted from the immunolocalization protocol (*d*). Magnification 200X.

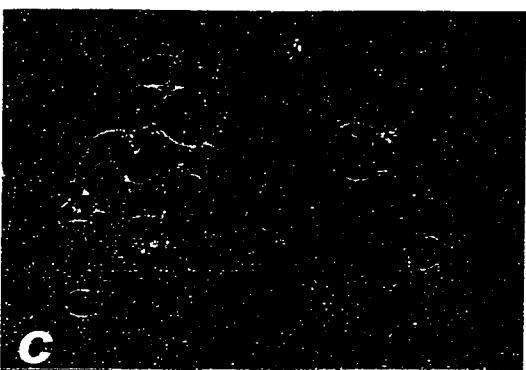
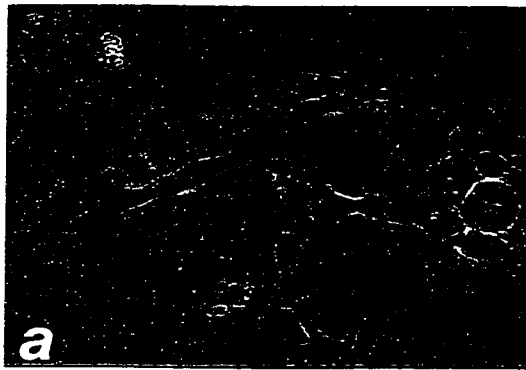


Figure 6

Northern blot analysis of the expression of c-*KIT* and SCF mRNAs in normal human OSE cells and epithelial ovarian tumors. Total cellular and tumor (T) RNA (15 µg of all samples except for ACC-LC-80 cells which had only 5 µg loaded since these cells overexpress c-*KIT* mRNA) were probed sequentially with a [³²P]-labelled human c-*KIT* cDNA (A), human SCF cDNA (B) and tubulin cDNA (to indicate levels of RNA loaded; C). ACC-LC-80 cells served as a positive control for c-*KIT* and SCF expression, OVCAR-3 cells served as a negative control for both c-*KIT* and SCF expression, and OVCA 420 cells were used as a positive and negative control for SCF and c-*KIT* expression, respectively. No 5.1 kb c-*KIT* mRNA was detected in normal human OSE cells, but all of the tumors on this blot expressed c-*KIT*, with expression levels ranging from quite low (T1.139, T1.143) to high (T1.138, T1.142). Both normal human OSE cells and the tumors expressed appreciable amounts of the 6 kb SCF mRNA.

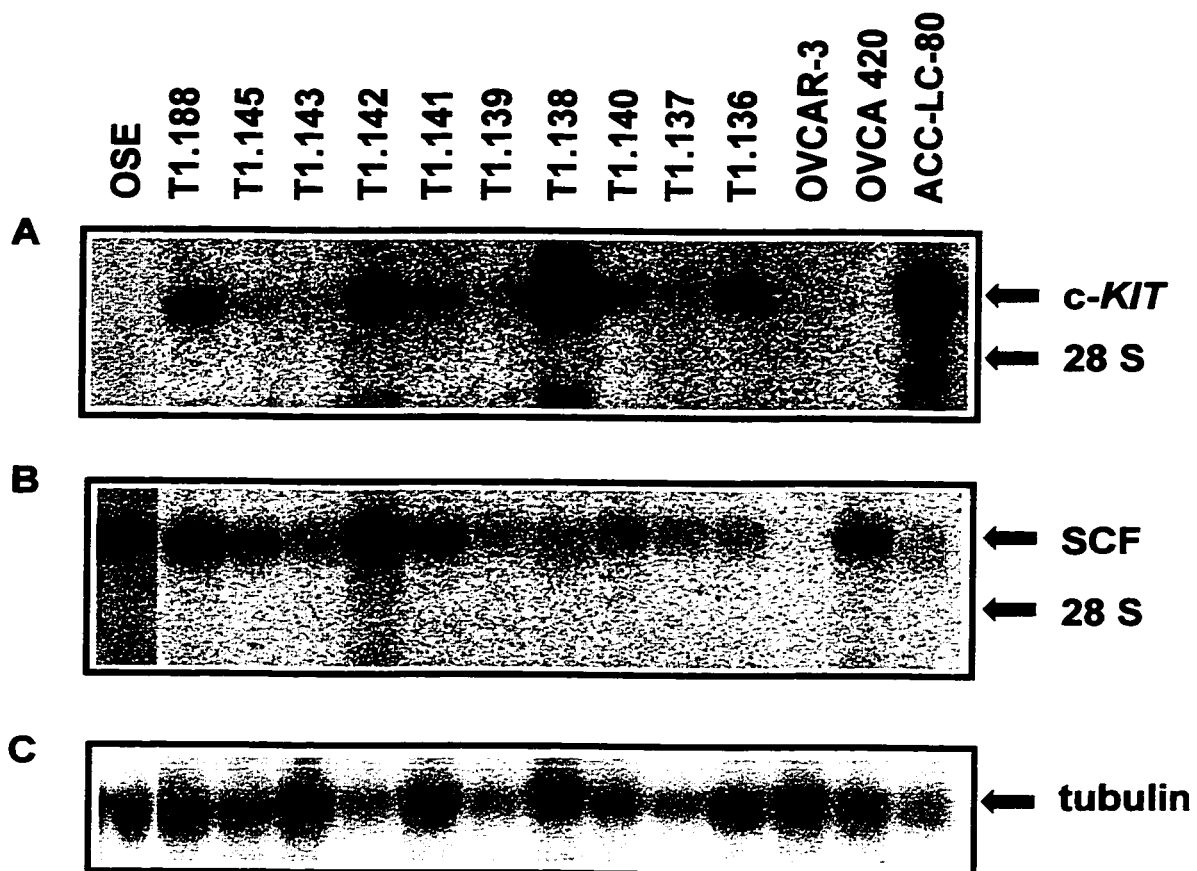
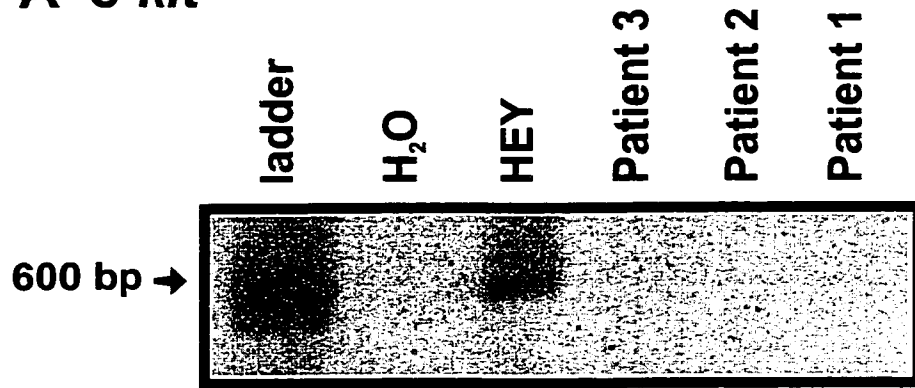


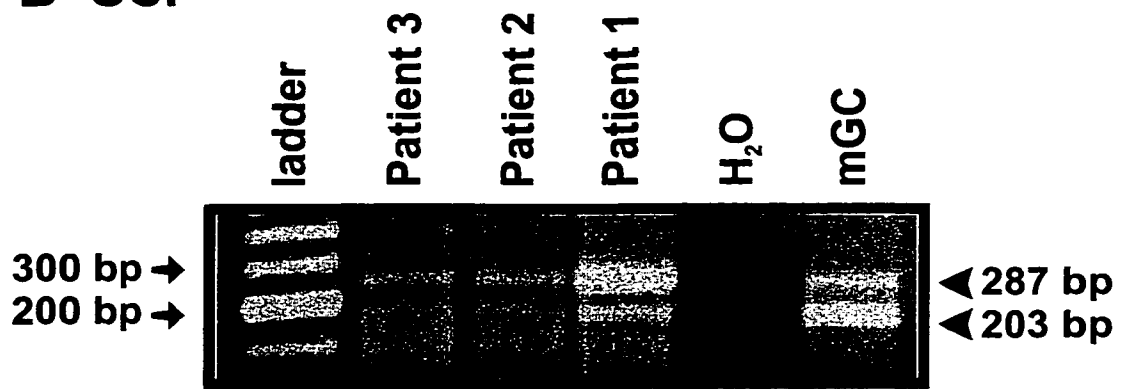
Figure 7

RT-PCR analysis of the expression of c-*KIT* and SCF mRNAs in normal human OSE cells. RT-PCR was performed on total RNA isolated from cultured normal OSE cells from three different patients. C-*KIT* primers were used to amplify an approximate 600 bp fragment (A), while SCF primers amplified 287 bp and 203 bp fragments representative of the soluble and membrane-associated isoforms, respectively, of SCF (B). HEY ovarian carcinoma cells served as the positive control for c-*KIT* expression, while murine granulosa cells (mGC) were used as the positive control for SCF expression. The patients' normal OSE cells did not express c-*KIT* mRNA, but they did express SCF, with relatively higher amounts of the soluble SCF isoform.

A c-kit



B SCF



RT-PCR analysis indicated that the OSE cells expressed relatively higher amounts of the transcripts encoding the soluble form of SCF (Figure 7).

Immunohistochemical detection of KIT protein in 6 pre- and 4 postmenopausal normal ovaries confirmed that intact normal OSE did not express KIT (Figure 8*a,b*). However, KIT protein was readily detectable in the epithelial cells of approximately 50% (Table 2) of the post-ovulatory epithelial invaginations (Figure 8*c*, arrows) and inclusion cysts (Figure 8*c*, arrowheads). In premenopausal ovaries, KIT protein was expressed by oocytes at all stages of follicular development, from primordial (Figure 8*a*) to antral-stage oocytes, with predominant membrane localization (Figure 8*a*, arrowheads) of KIT as well as cytoplasmic expression of the protein. In one premenopausal ovary, staining for KIT protein was also evident in a zone of transitional cell metaplasia (Figure 8*d*). Additionally, tissue mast cells showed intense membrane staining of KIT protein, with some cytoplasmic localization (Figure 8*d*; confirmation of the mast cell type was done by toluidine blue staining). Homogeneous expression of KIT protein was also detected in granulosa-luteal cells of corpora lutea in normal ovaries (Figure 8*e*, CL).

3.3.2 Expression of KIT and SCF in epithelial ovarian tumors

In contrast with the normal OSE cells which did not express *c-KIT*, evaluation of 50 epithelial ovarian tumors indicated that 76% (38/50, Table 3) of these tumors expressed *c-KIT* mRNA (5.1 kb transcript, Figure 6A). Data indicated that 87% (n=15) of benign and LMP tumors expressed *c-KIT*, whereas expression was less frequent in malignant tumors (71%, n=35). Additionally, 92% (35/38) of the *c-KIT*-expressing tumors also expressed SCF

Table 2

Frequency of expression of KIT receptors in the OSE cells of surface invaginations and inclusion cysts of healthy ovaries from pre- and postmenopausal women.

Ovary Status	N	Mean No. Inclusion Cysts (range)	% KIT ⁺ Inclusion Cysts	Mean No. Invaginations (range)	% Kit ⁺ Invaginations
Premenopausal	6	6 (0-21)	77	3 (0-7)	44
Postmenopausal	4	7 (1-19)	52	8 (2-10)	50

Figure 8

Immunohistochemical detection of KIT protein in normal human ovaries. Paraffin-embedded tissue sections were stained for KIT by the immunoperoxidase method using a polyclonal anti-KIT antibody targetted against the carboxy-terminal domain of the KIT receptor, and counterstained using hematoxylin (blue). Positive KIT immunoreactivity is seen as a brown reaction product. KIT protein is detected on the membrane (arrowheads) and in the cytoplasm of oocytes (O), but not in the OSE cells on the far right side of panel *a* and left side of panel *b*. A post-ovulatory epithelial invagination (arrows) and an epithelial inclusion cyst (IC; arrowheads) both show significant KIT staining in the cytoplasm of the epithelial cells (*c*). Intense membrane localization of KIT protein is evident in mast cells (MC), and KIT is also observed in a zone of transitional cell metaplasia (TM; *d*). A corpus luteum shows homogeneous expression of KIT (CL; *e*). Scale bar: 100 μ M.

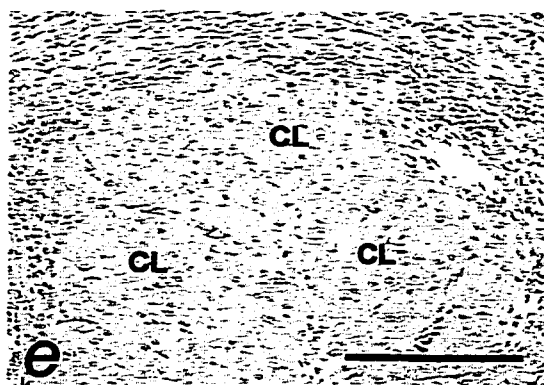
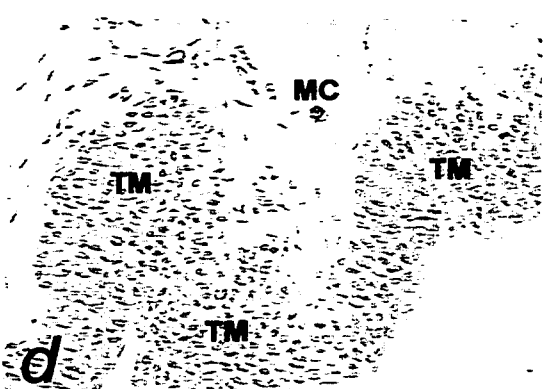
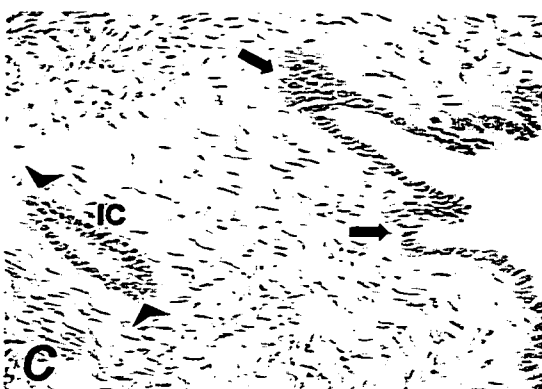
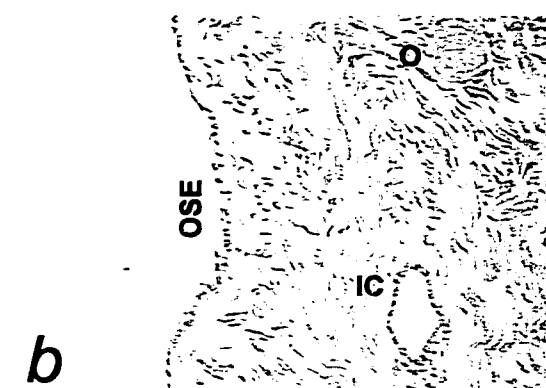


Table 3

Expression of c-*KIT* and SCF in human epithelial ovarian tumors classified according to tumor subtype.

Tumor subtype	Number of tumors	Expressing c- <i>KIT</i> (%)	Expressing SCF (%)	Expressing c- <i>KIT</i> and SCF (%)
Benign	7	5 (71)	6 (86)	5 (71)
LMP ¹	8	8 (100)	7 (88)	7 (88)
Malignant	35	25 (71)	25 (71)	23 (66)
serous	25 ²	19 (76)	17 (68)	17 (68)
mucinous	1	0 (0)	1 (100)	0 (0)
endometrioid	5	3 (60)	3 (60)	3 (60)
clear cell	4	3 (75)	3 (100)	3 (75)
Total	50	38 (76)	38 (76)	35 (70)

Values were compiled from the results of RNA and protein analyses. ¹LMP means a tumor of low malignant potential. ²This category includes one tumor that was a mixture of serous and endometrioid, one serous tumor that contained focal clear cell differentiation, and one tumor that was unclassified but probably serous.

mRNA (Figure 6B, Table 3).

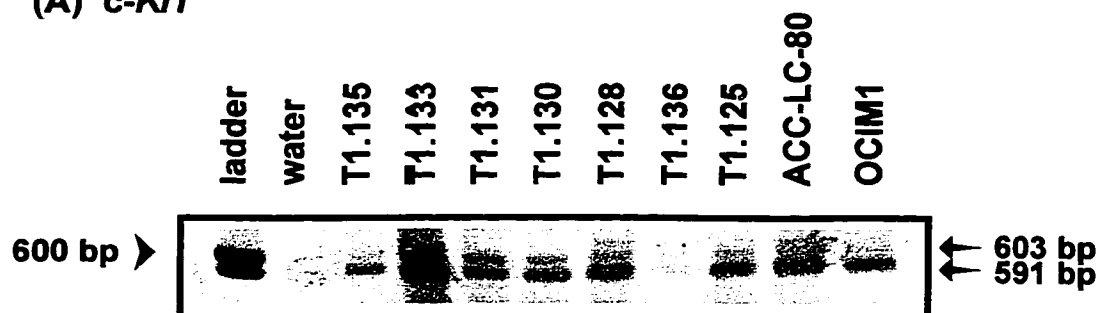
RT-PCR analysis demonstrated that the tumors produced *c-KIT* transcripts that could encode both the membrane-associated and soluble forms of the KIT receptor (591 bp and 603 bp, respectively; Figure 9A). The majority of the tumors did, however, produce higher amounts of the transcripts encoding the membrane-associated isoform of KIT relative to the soluble isoform. Confirmation that the tumor PCR fragments were truly *c-KIT* was done by digestion and sequencing of the 603 bp transcripts encoding the soluble form of KIT, which demonstrated 98% and 97% homology to the human *c-KIT* sequence (Yarden *et al.*, 1987) for the 229 bp (199 of 203 bp sequenced) and 374 bp (280 of 289 bp sequenced) fragments, respectively. RT-PCR analysis also showed that the tumors produced transcripts encoding both the membrane-associated and soluble isoforms of SCF (203 bp and 287 bp, respectively), with relatively higher expression of the soluble isoform (Figure 9B).

In order to confirm that tumors which expressed *c-KIT* mRNA also expressed KIT protein, western blot analysis was done on 20 of the 50 tumors analyzed in this study. As demonstrated in Figure 10B, KIT proteins having the expected molecular masses of 160, 145 (T1.117 and T1.186) and/or 125 kDa (T1.187) were routinely detected in the tumors. Tumors T1.112 and T1.118, which did not express *c-KIT* mRNA (Figure 10A), also did not express KIT protein; indeed, for the subset of tumors that were examined for KIT protein expression by western blot analysis, there was always concordance between the *c-KIT* mRNA expression and KIT protein expression. A band of approximately 100 kD was routinely detected in the tumor tissues, but this band was found to be due to non-specific binding of the secondary antibody since western blots that were probed with *only* the

Figure 9

RT-PCR analysis of the expression of membrane-associated and soluble c-*KIT* and SCF isoforms in human epithelial ovarian tumors. RT-PCR was performed on total RNA isolated from human cell lines and epithelial ovarian tumors (T). *C-KIT* primers were used to amplify the 603 bp and 591 bp fragments representative of the soluble and membrane-associated isoforms, respectively, of the KIT receptor (A). SCF primers were used to amplify the 287 bp and 203 bp fragments representative of the soluble and membrane-associated isoforms, respectively, of SCF (B). ACC-LC-80 small cell lung cancer cells and OCIM1 erythroleukemia cells served as positive controls for the expression of c-*KIT* isoforms, while ROSE 199 cells were used as the positive control for SCF expression. The tumors expressed relatively higher amounts of the transcripts encoding the membrane-associated isoform of c-*KIT* and the soluble isoform of SCF.

(A) c-KIT



(B) SCF

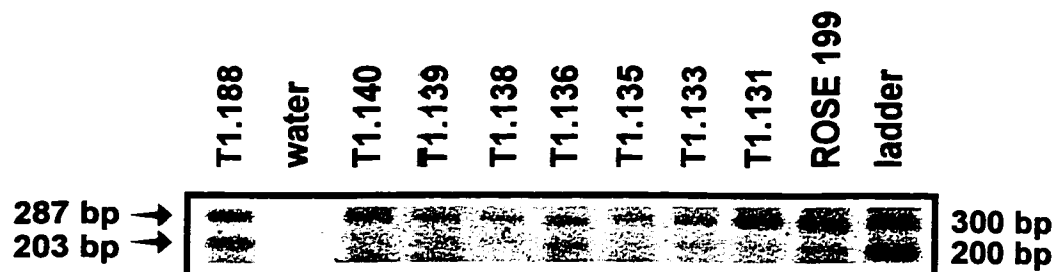
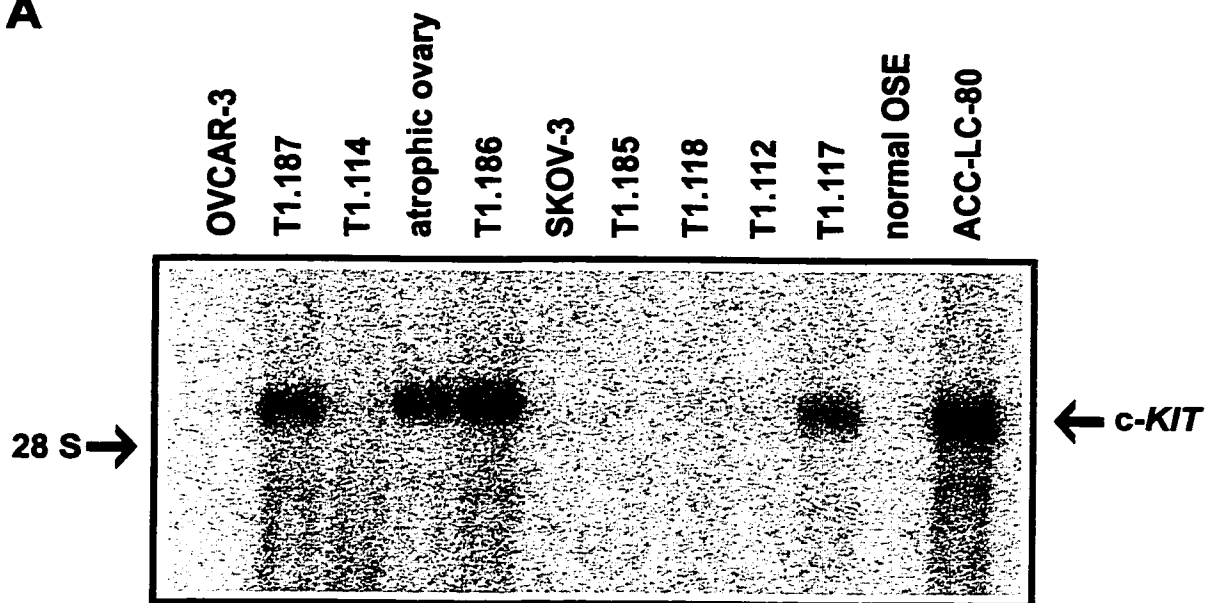


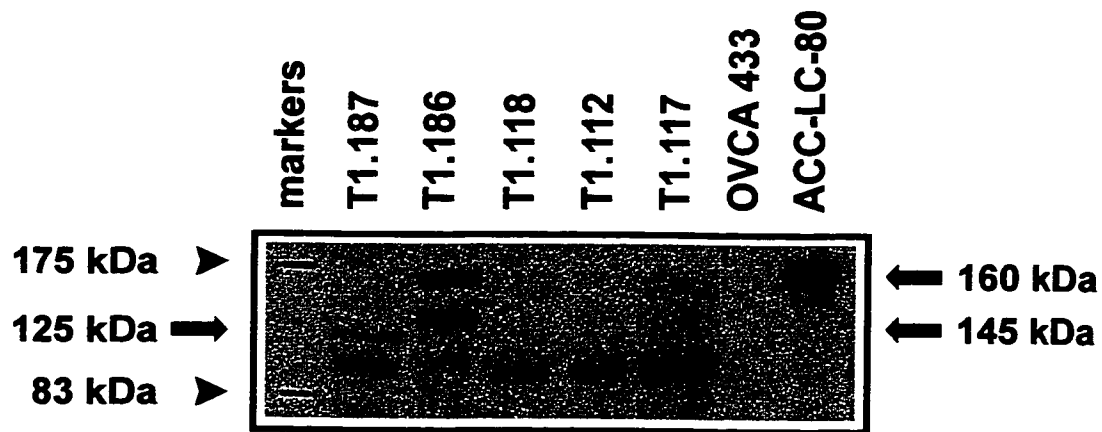
Figure 10

Correlation of c-*KIT* mRNA with KIT protein expression in human epithelial ovarian tumors. For northern blot analysis (A) of the expression of c-*KIT* mRNA in human epithelial ovarian tumors, total cellular and tumor (T) RNA (15 µg of each sample except for ACC-LC-80 cells which had only 5 µg loaded since these cells overexpress c-*KIT* mRNA) were probed with a [³²P]-labelled human c-*KIT* cDNA. ACC-LC-80 small cell lung cancer cells served as the positive control for c-*KIT* expression, while SKOV-3 ovarian carcinoma cells were used as the negative control. Western blot analysis (B) to determine the expression of KIT protein in 5 of the tumors shown in (A) was done using total cellular (50 µg) or tumor (25 µl of a 1 ml lysate, corresponding to 278 µg of T1.187, 165 µg of T1.186, 418 µg of T1.118, 330 µg of T1.112 and 425 µg of T1.117) protein lysates and an anti-KIT MAbs. ACC-LC-80 cells again served as the positive control for KIT protein expression, while OVCA 433 ovarian carcinoma cells were used as a negative control. The sizes of the molecular weight protein markers are indicated by the arrowheads on the left side of the blot. C-*KIT* mRNA was detected in tumors T1.187, T1.186, and T1.117 and KIT proteins with molecular masses of approximately 160, 145 (T1.117, T1.186) or 125 kDa (T1.187) were also detected in those tumors. Tumors T1.112 and T1.118 which show no detectable KIT protein expression also did not express c-*KIT* mRNA. The approximately 100 kDa band detected in the tumors is attributable to non-specific binding of the secondary antibody.

A



B



secondary antibody showed immunodetection of the 100 kD band.

3.3.4 Immunohistochemical detection of KIT protein in epithelial ovarian tumors

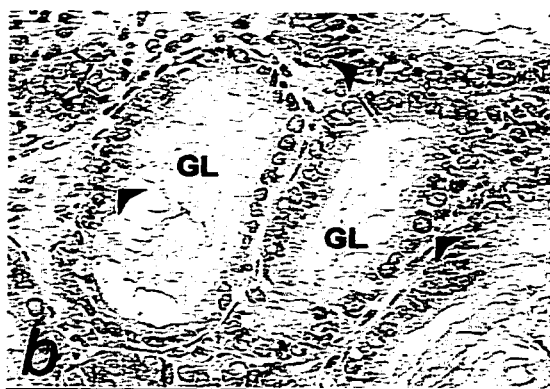
In order to confirm that the *c-KIT* mRNA and KIT protein detected in the ovarian tumors by northern, RT-PCR and western analyses was actually produced by the OSE-derived tumor cells, immunohistochemical localization of KIT protein in representative histological subtypes of the ovarian tumors (Figure 11) was performed using an immunoperoxidase staining method. KIT protein was clearly evident in the basally-located cytoplasm of columnar epithelial cells in a mucinous LMP tumor (Figure 11*a*, arrowheads), with some light staining in the stromal tissue as well. KIT staining was observed predominantly in the cytoplasm in the glandular epithelium of a malignant endometrioid tumor (Figure 11*b*, arrowheads). Nests of tumor cells in a malignant clear cell tumor showed moderate to strong staining for KIT in all of the tumor cells (Figure 11*c*). Similarly, moderate to intense KIT staining in the cytoplasm (and an occasional nucleus) was also evident in the majority of tumor cells of a malignant serous tumor (Figure 11*d*).

Thus, KIT protein was detected in OSE-derived tumor cells. However, the protein was not localized to the membrane of the tumor cells as would be expected for this growth factor receptor and as was seen for the normal cells (i.e., oocytes and mast cells) in the healthy ovaries; instead, KIT protein in the tumor cells was found to have a predominantly cytoplasmic localization.

Figure 11

Immunohistochemical detection of KIT protein in human epithelial ovarian tumors.

Paraffin-embedded tissue sections were stained for KIT by the immunoperoxidase method using a polyclonal anti-KIT antibody targetted against the carboxy-terminal domain of the KIT receptor, and counterstained using hematoxylin (blue). Positive KIT immunoreactivity is seen as a brown reaction product. A mucinous tumor of low malignant potential shows KIT staining basally in the cytoplasm of the epithelial cells and lightly in the stroma (S; *a*). KIT staining is seen in some of the epithelial cells lining glands (GL) in a malignant endometrioid tumor (*b*). Nests of tumor cells (TC) in a malignant clear cell tumor all express abundant KIT, while the stromal cells (S) do not (*c*). Moderate to strong KIT positivity is seen predominantly in the cytoplasm (with some nuclear staining) of the majority of tumor cells in a malignant serous tumor (*d*). Lack of KIT staining in a consecutive section of the same tumor as in *d*, for which the primary antibody was omitted, indicates the specificity of the observed KIT staining (*e*). Scale bar: 100 μ M.



3.3.5 Correlation of KIT expression with histologic grade and stage of the tumors and clinical disease progression

There was no obvious correlation between c-*KIT* expression and histologic grade of the malignant tumors (Table 4). The relative amount of c-*KIT* mRNA expressed by well differentiated (low grade) vs. poorly differentiated (high grade) tumors was not significantly different, as determined by densitometric analysis of the northern blots (data not shown). However, there was a decrease in, or lack of, KIT expression in advanced stage malignant tumors, i.e., 100% of LMP tumors expressed c-*KIT* compared to 81% of malignant stage III tumors and 0% of malignant stage IV tumors (Table 5). Correlation of c-*KIT* expression with clinical disease progression in 34 of 35 patients with malignant tumors for whom follow-up information was available (Table 6) indicated that 9 patients had tumors that did not express KIT, 8 (89%) of whom have died and the remaining 1 has recurrent disease. Of 25 patients with tumors expressing KIT, 56% are still alive and 8 of these patients have no evidence of disease. Statistical comparison of both the overall and the disease-free survival of patients whose tumors did versus did not express c-*KIT* indicated that there was a difference of 8 months in the median overall survival time of the patients (23 months for patients with c-*KIT*-negative tumors vs. 31 months for patients with c-*KIT*-positive tumors; Figure 12A), but this difference did not achieve statistical significance. However, the disease-free patient survival, i.e., the elapsed time following surgery before the disease recurred, was significantly longer ($p<0.05$) for patients whose tumors expressed c-*KIT* compared with those patients who had c-*KIT*-negative tumors [median disease-free survival was 12 months for patients with c-*KIT*-negative tumors, while, with a median follow-up time

Table 4

Expression of c-*KIT* and SCF in malignant epithelial ovarian tumors according to the grade of the tumor.

Histologic tumor grade	Number of tumors	Expressing c- <i>KIT</i> (%)	Expressing SCF (%)	Expressing c- <i>KIT</i> and SCF (%)
1	4	3 (75)	3 (75)	3 (75)
2	3	2 (67)	2 (67)	2 (67)
2/3 and 3	28	20 (71)	20 (71)	18 (64)

Values were compiled from the results of RNA and protein analyses of malignant tumors. Histologic grade was assigned based on the permanent section tumor morphology. Grade 1 refers to a well differentiated tumor, grade 2 indicates a moderately differentiated tumor and grade 3 refers to a poorly differentiated tumor.

Table 5

Expression of c-*KIT* and SCF in tumors of low malignant potential and malignant tumors according to the stage of the tumor.

Tumor stage ¹	Number of tumors	Expressing c- <i>KIT</i> (%)	Expressing SCF (%)	Expressing c- <i>KIT</i> and SCF (%)
LMP ² , stage I	6	6 (100)	5 (83)	5 (83)
LMP, stage III	2	2 (100)	2 (100)	2 (100)
malignant, stage I	4	3 (75)	4 (100)	3 (75)
malignant, stage III	27	22 (81)	20 (74)	20 (74)
malignant, stage IV	4	0 (0)	0 (0)	0 (0)

Values were compiled from the results of RNA and protein analyses. ¹Tumors were staged at the time of surgical removal according to the FIGO staging system. ²LMP means a tumor of low malignant potential.

Table 6

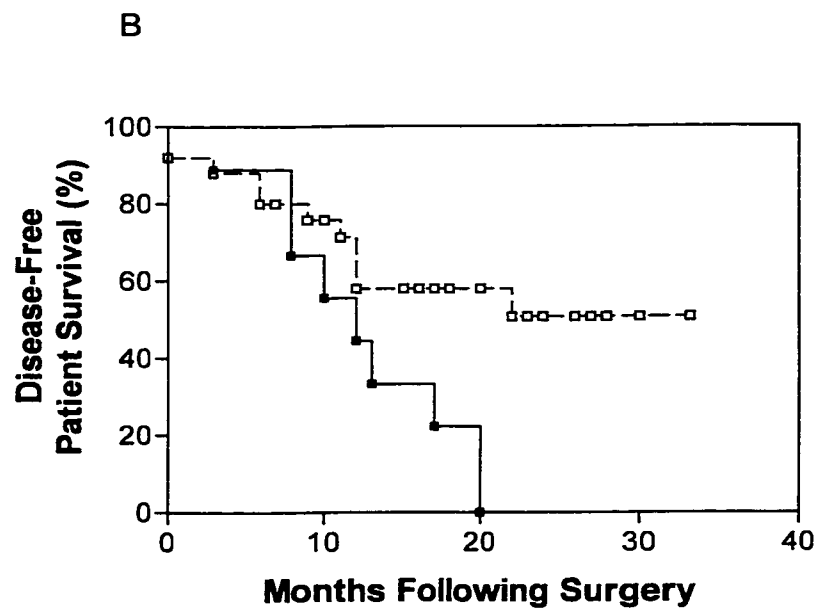
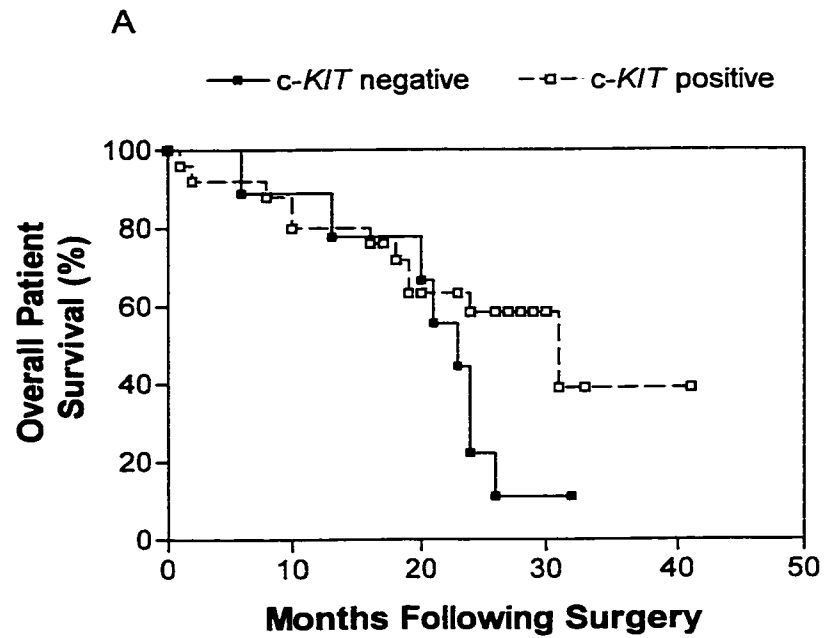
Correlation of expression of c-*KIT* in malignant epithelial ovarian tumors with clinical disease progression.

Disease status	Number of patients ¹	KIT-positive tumors (%)	KIT-negative tumors (%)
No evidence of disease	8	8 (100)	0 (0)
Alive with disease	7	6 (86)	1 (14)
Dead of disease	19	11 (58)	8 (42)

Values were compiled from the results of RNA and protein analyses and refer to disease progression (median follow-up time of 24 months) in patients following surgical removal of malignant tumors. ¹Data are presented for 34 of 35 patients with malignant tumors, as 1 patient was lost to follow-up.

Figure 12

Correlation of patient survival with epithelial ovarian tumor *c-KIT* status. Shown are Kaplan-Meier plots comparing the overall survival (A) and disease-free survival (B) of 34 patients with malignant tumors that did (open symbols) or did not (closed symbols) express *c-KIT*. There was no significant difference in the overall survival time of patients with *c-KIT*-negative or *c-KIT*-positive tumors, i.e., the median survival time for patients with *c-KIT*-negative tumors was 23 months versus 31 months for patients with *c-KIT*-positive tumors (A). However, there was a statistically significant ($p<0.05$) decrease in the disease-free survival time of patients with *c-KIT*-negative tumors (median disease-free survival 12 months) compared with patients with *c-KIT*-positive tumors [with a median follow-up time of 15 months (range 0-33 months), the median disease-free survival time has not been reached].



of 15 months (range 0-33 months) for patients with c-*KIT*-positive tumors, the median disease-free patient survival has not been reached; Figure 12B].

3.4 Discussion

The expression of c-*KIT* and SCF in epithelial ovarian cancers has been examined previously (Inoue *et al.*, 1994; Wrigley *et al.*, 1996; Arber *et al.*, 1998), but with conflicting results. We have confirmed that epithelial ovarian tumors frequently express both c-*KIT* and SCF, and have demonstrated differential expression in benign, LMP and malignant tumors. Normal OSE cells express SCF, but not c-*KIT*, whereas distinct KIT expression can frequently be found in the epithelial cells of post-ovulatory invaginations and inclusion cysts in healthy ovaries. Within the epithelial ovarian tumors, moderate to strong KIT expression was clearly detected in the OSE-derived tumor cells. Most remarkably, these results suggest that women with malignant ovarian tumors that do not express c-*KIT* have a less favorable prognosis than those with malignant tumors expressing c-*KIT*.

Both c-*KIT* and SCF are expressed in normal human ovaries. KIT receptors are found on oocytes at all stages of follicular development in mouse (Manova *et al.*, 1990; Orr-Urtreger *et al.*, 1990; Horie *et al.*, 1991), rat (Ismail *et al.*, 1997), and human (Horie *et al.*, 1993 and this study). SCF is produced by the granulosa cells in mouse (Manova *et al.*, 1993; Motro and Bernstein, 1993), rat (Ismail *et al.*, 1996), and human (Laitinen *et al.*, 1995), and it has been implicated in controlling the function of the ovarian follicle during the human menstrual cycle (Laitinen *et al.*, 1995), as well as regulating mouse oocyte growth (Packer *et al.*, 1994) and rat oocyte meiotic progression (Ismail *et al.*, 1996; Ismail *et al.*, 1997).

As was recently shown for rat OSE cells (Ismail *et al.*, 1999), in this study normal human OSE cells expressed SCF but not *c-KIT*. Lack of expression of *c-KIT* in normal human OSE cells had been previously mentioned (Lammie *et al.*, 1994), however, during the writing of this thesis Parrott *et al.* (2000) reported that normal human OSE cells did express *c-KIT*. The reason for our conflicting results is not clear at this time.

In contrast to the surface epithelial cells, the results of this study demonstrated that relocation of these cells into the stromal compartment as invaginations or inclusion cysts, as can happen after ovulation, frequently resulted in the induction of KIT expression. The mechanism by which *c-KIT* expression was turned on may be related to the greater contact of the epithelial cells with ovarian stroma or the accumulation of regulatory factors in the lumen of the inclusion cyst. Stimulation of KIT expression has been achieved in myeloid leukemia cells by addition of GM-CSF (Brizzi *et al.*, 1993), in bone marrow cells by IL-1 treatment (Neta *et al.*, 1994), and in teratocarcinoma cells by activation of the cAMP signalling pathway (Nishina *et al.*, 1992). Detection of KIT protein in the P₄-producing granulosa-luteal cells of corpora lutea in normal ovaries in this study also suggests that P₄ might upregulate *c-KIT* expression *in vivo*. Whether expression of KIT in the epithelial inclusion cysts has the potential to be oncogenic is not known; however, there is evidence that epithelial ovarian carcinomas can arise *de novo* from surface epithelial inclusions, and tubal metaplasia of inclusion cysts in ovaries contralateral to those containing epithelial cancers is significantly more common than in control ovaries (Bell and Scully, 1994). It is interesting in this regard that expression of KIT was detected in a zone of transitional cell metaplasia in a healthy ovary in this study.

Inoue *et al.* (1994) first reported the co-expression of c-*KIT* and SCF in gynecological tissues and tumors. Surprisingly, none of the 4 benign epithelial ovarian tumors reported by that group expressed KIT or SCF, whereas 5 of 7 benign tumors in this study showed immunodetectable KIT, and all of these co-expressed SCF. Similarly, the frequency of KIT expression in malignant tumors in the previous report was 17% (n=18) compared to 71% (n=35) in this study. Wrigley *et al.* (1996) failed to detect KIT expression in any (n=38) epithelial ovarian cancers or in 3 benign tumors. Both of these previous studies used different immunohistochemical procedures, as well as different anti-KIT antibodies, which may account for differences in the ability to detect KIT in the tumors. In accordance with the results presented here, KIT expression has been detected in 87% of epithelial ovarian cancers (Arber *et al.*, 1998).

KIT expression has been demonstrated in a variety of solid tumors in addition to ovarian cancer, including breast cancer (Hines *et al.*, 1995), germ cell cancer (Strohmeyer *et al.*, 1991), melanoma (Natali *et al.*, 1992c), and small cell lung cancer (Hibi *et al.*, 1991). In breast, small cell lung and ovarian cancers, co-expression of KIT and SCF has been reported, raising the possibility of autocrine growth regulation. Indeed, such KIT-SCF autocrine interactions have already been shown to enhance breast (Hines *et al.*, 1995) and small cell lung (Krystal *et al.*, 1996) tumor cell proliferation *in vitro* and testicular tumor cell proliferation *in vivo* (Kondoh *et al.*, 1995). Therefore, it is possible that the high incidence of KIT receptor expression in benign and LMP ovarian tumors, arising in a cell type that normally expresses SCF, may contribute to the uncontrolled proliferation of these tumors.

While KIT activity may help to drive cell proliferation in many types of cancers,

malignant transformation and disease progression has been associated with a loss of KIT protein expression in some tissue types. For example, KIT expression positively correlates with growth control or differentiation of normal breast epithelium, but malignant transformation is associated with loss of expression of this protein (Natali *et al.*, 1992b). While c-KIT is expressed in normal thyroid and most benign lesions, the receptor is undetectable in the majority of malignant thyroid tumors (Natali *et al.*, 1995). Similarly, the progression of melanoma has been associated with a loss of c-KIT expression (Natali *et al.*, 1992c), which correlated with a higher metastatic potential in nude mice (Huang *et al.*, 1996). In our study, the decreased expression of c-KIT in malignant stage III tumors relative to benign and LMP tumors, and the lack of c-KIT expression in any of the malignant stage IV ovarian tumors, suggests that progression of epithelial ovarian cancer may also be associated with loss of c-KIT expression. Changes in the functional response to KIT activation in cells during transformation and disease progression may be attributed to levels of available ligand or alterations in the KIT signal transduction pathway. Indeed, loss of expression of AP-2, a transcription factor that regulates c-KIT expression, appears to be a crucial event in the development of malignant melanoma (Huang *et al.*, 1998).

In this study, evaluation of ovarian cancers by RT-PCR analysis revealed that the tumor cells primarily produced transcripts encoding the membrane-associated receptors, but transcripts encoding the isoform of KIT that yields soluble receptors were also present in the tumors. The presence of transcripts for s-KIT suggests that ovarian cancers can potentially produce s-KIT as a biomarker. S-KIT is detectable in human serum at a concentration of 325 ng/ml (Wypych *et al.*, 1995), however, patients with acute and chronic myeloid leukemia

were found to have elevated serum levels of s-KIT (Kawakita *et al.*, 1995). Although little is known about the functional role of s-KIT, serum levels of s-KIT appear to be related to the stage of differentiation of AML blasts in accordance with their expression of c-KIT, and this phenomenon has been demonstrated to be useful for assessment of leukemic cell burden (Tajima *et al.*, 1998). The possible production of s-KIT by ovarian cancers, as well as the possibility that this production is reflected in serum levels of the protein, remain to be determined.

Establishing the function of KIT in ovarian cancer cells may prove challenging since, despite the relative abundance of the transcripts encoding membrane-associated KIT, tumor cells had cytoplasmic KIT expression rather than the normal localization of KIT on the cell surface. Cytoplasmic localization of immunoreactive KIT protein has previously been reported in breast cancer cells (Hines *et al.*, 1995), gliomas (Stanulla *et al.*, 1995), germ cell cancers (Izquierdo *et al.*, 1995) and a variety of other cancers (Arber *et al.*, 1998). The failure of KIT to localize to the cell membrane probably accounts for the inability to demonstrate a mitogenic effect when SCF was added to glioma cells, and the inability to modulate glioma cell proliferation using antibodies that blocked KIT:SCF interactions (Stanulla *et al.*, 1995). It is possible that the KIT:SCF interaction may mediate non-proliferative signals or may employ intracellular mechanisms for autocrine growth regulation of cancer cells.

The prognostic significance of the expression of growth factors or cytokines and their receptors in ovarian cancers is quite variable. For example, strong expression of epithelial CSF-1 and c-FMS in the metastases of ovarian cancer patients appeared to be an independent

predictor of poor clinical outcome in one study (Chambers *et al.*, 1997). Activating KIT mutations have been shown to predict poor prognosis in gastrointestinal stromal/smooth muscle tumors (Ernst *et al.*, 1998). In AML patients there is a correlation between c-*KIT* expression and the multi-drug resistance phenotype, which may be a consequence of co-expression at a similar stage of differentiation, and which may account for the association of high KIT expression with poor outcome (Sincock and Ashman, 1997). Conversely, a correlation between KIT expression in malignant epithelial ovarian cancers and increased disease-free survival of patients has been reported herein. In accordance with this data, patients whose tumors retained PDGF receptor staining positivity were shown to have a prolonged relapse-free survival (Dabrow *et al.*, 1998). The mechanism underlying a prognostic significance of KIT expression in epithelial ovarian cancers is explored in Chapters 4 and 5.

Chapter 4 - Expression and regulation of c-*KIT* and SCF in human ovarian carcinoma cells

4.1 Introduction

The results of Chapter 3 indicated that the c-*KIT* protooncogene was not expressed in normal human OSE cells, but it was frequently expressed in OSE-lined inclusion cysts and epithelial ovarian tumors. Since expression of c-*KIT* in the tumors correlated with disease progression and patient survival, it is pertinent to identify intraovarian growth regulatory factors which may also regulate c-*KIT* expression in ovarian cancer cells, and to determine whether activated KIT can affect the proliferation or survival of these cells.

The risk of developing ovarian cancer increases significantly after menopause, so it has been suggested that the hormonal changes associated with menopause may contribute to alterations in the expression and/or activity of protooncogenes and growth factors, thereby promoting ovarian tumorigenesis (Cramer and Welch, 1983). Normal OSE cells have receptors for gonadotropins (Zheng *et al.*, 1996; Konishi *et al.*, 1999) and respond to gonadotropin treatment with increased proliferation (Osterholzer *et al.*, 1985b; Konishi *et al.*, 1999). Gonadotropin involvement in ovarian cancer has been demonstrated by the ability of FSH and LH to stimulate ovarian cancer cell proliferation *in vitro* (Simon *et al.*, 1983; Wimalasena *et al.*, 1993) and *in vivo* (Wimalasena *et al.*, 1992; Kurbacher *et al.*, 1995; Schiffenbauer *et al.*, 1997), and to promote angiogenesis in transplanted ovarian carcinoma *in vivo* (Schiffenbauer *et al.*, 1997). Furthermore, both c-*kit* and SCF expression in the ovary have been found to be regulated by gonadotropins (Motro and Bernstein, 1993; Laitinen *et al.*,

1995; Ismail *et al.*, 1996).

Growth factor regulation of the proliferation of normal OSE cells and ovarian cancer cells is well documented. Normal OSE cells express EGF-R (Berchuck *et al.*, 1991) and are growth-stimulated by EGF/TGF- α (Siemens and Auersperg, 1988; Rodriguez *et al.*, 1991; Jindal *et al.*, 1994). Similarly, the proliferation of most ovarian carcinoma cells is enhanced in the presence of EGF/TGF- α (Berchuck *et al.*, 1992; Stromberg *et al.*, 1992; Wimalasena *et al.*, 1992; Jindal *et al.*, 1994; Simpson *et al.*, 1998). Normal OSE cells and ovarian cancer cells also produce TGF- β which suppresses their growth (Berchuck *et al.*, 1992; Hurteau *et al.*, 1994; Havrilesky *et al.*, 1995; Kim *et al.*, 1997). At the molecular genetic level, these growth factors have been shown to regulate HER-2/*neu* and *c-myc* gene expression in ovarian cancer cells (Marth *et al.*, 1992; Kim *et al.*, 1997; Park, 1997). Additionally, TGF- β regulation of *c-KIT* and SCF has been reported in a number of normal cells (Dubois *et al.*, 1994; Heinrich *et al.*, 1995; Sansilvestri *et al.*, 1995; Ismail *et al.*, 1997; Sugimoto *et al.*, 1999) and transformed cells (de Vos *et al.*, 1993; Bellone *et al.*, 1997).

The objectives of this study were: (1) to identify human ovarian carcinoma cells that express *c-KIT* and SCF; (2) to assess whether intraovarian factors that regulate ovarian cancer cell proliferation also affect *c-KIT* and SCF expression in those cells; (3) to determine whether disruption or stimulation of the KIT:SCF interaction in ovarian carcinoma cells can influence proliferation; and (4) to examine the subcellular localization of KIT protein in ovarian carcinoma cells.

4.2 Materials and Methods

4.2.1 Determination of c-*KIT* and SCF mRNA and KIT protein expression in human ovarian carcinoma cells

Twelve different human ovarian carcinoma cell lines were screened for their expression of c-*KIT* and SCF mRNAs by northern blot analysis. Cultured cells were harvested at approximately 70-80% confluence, and their cellular RNA was extracted and probed for c-*KIT* and SCF mRNAs as described in section 2.3. Confirmation that c-*KIT* mRNA-expressing ovarian carcinoma cells also expressed KIT protein was done by harvesting the cells and performing western blot analysis on total cellular protein lysates as described in section 2.5.

4.2.2 Expression of gonadotropin receptors in HEY and OVCA 429 cells

Northern blot analysis to determine the expression of FSH-R and LH-R mRNAs in HEY and OVCA 429 cells, as well as primary rat OSE cells and ROSE 199 cells, was done as described for the c-*KIT* and SCF studies (section 2.3) except using a [³²P]-labelled 622 bp rat LH-R cDNA fragment or a 477 bp rat FSH-R cDNA fragment (the plasmids containing these cDNAs were generously provided by D. Segaloff, University of Iowa, Iowa City, IA).

4.2.3 Culture of HEY cells in the presence of dbcAMP and growth factors

HEY cells were seeded at 5×10^4 cells in triplicate 60 mm dishes in α -MEM + 5% FCS (base medium), and were initially incubated for 2 h at 37°C in the absence of additives

to allow for cell attachment to the dishes. The media was then replaced with base medium (control) or base medium supplemented with either 2 mM dbcAMP (Boehringer Mannheim, Germany), or 10 ng/ml recombinant human TGF- α (R&D Systems, Minneapolis, MN), or 10 ng/ml of recombinant human TGF- β_1 (R&D Systems), or dbcAMP and TGF- α or - β together, and the cells were cultured for an additional 48 h. The dbcAMP was sterily prepared as a 40 mM stock solution in base medium and was diluted directly into the experimental culture media. The dbcAMP stock was stored at 4°C for a maximum of two months. TGF- α was prepared as a sterile stock solution of 10 ng/ μ l in 10 mM acetic acid, 0.1% BSA and was stored at -20°C for several months. TGF- β was sterily prepared in 4 mM HCl, 0.1 % BSA to yield a 20 ng/ μ l stock solution (also stored at -20°C), which was diluted 40-fold into the culture medium in order to minimize any potentially toxic effects of the HCl. (Preliminary studies of HEY cells cultured in medium containing similarly diluted 4 mM HCl in the absence of TGF- β had ensured the absence of such toxicity). All of the dishes were coded by a second party to allow for blinded assessment. After 48 h of culture, HEY cell proliferation was assessed by counting the cells using hemacytometry as described in section 2.2.1. Subsequently, the triplicate cell samples from each treatment group were pooled and the cellular RNA was extracted for northern blot analysis of *c-KIT* and SCF expression as described in section 2.3, and for RT-PCR analysis to examine the relative abundance of the membrane-associated and soluble forms of both *c-KIT* and SCF as described in section 2.4. Densitometric analysis of the northern blots was carried out as described in section 4.2.6 below.

In a separate experiment, HEY cells were cultured for 24 h in the absence or presence

of 2 mM dbcAMP (5x100 mm dishes per treatment), and immunoprecipitation and western blot analysis of KIT protein expression was performed as described in section 2.4.

4.2.5 Expression of *c-KIT* and SCF in HEY and OVCA 429 cells at various confluencies

HEY and OVCA 429 cells were seeded at 1×10^5 cells or 2×10^5 cells, respectively, in duplicate or triplicate 100 mm dishes. Both cell lines were cultured in their regular maintenance media (Table 1) for up to 96 h. At 24, 48, 72, and 96 h post-seeding, the confluency of the cells in the dishes was estimated by microscopic observation, and the cells were counted using hemacytometry as described in section 2.2.1. Subsequently, the cellular RNA was extracted for northern blot analysis to determine the expression of *c-KIT* and SCF mRNAs as described in section 2.3 and for RT-PCR analysis to examine the relative abundance of the membrane-associated and soluble forms of both *c-KIT* and SCF as described in section 2.4. As an internal control, a third set of primers was used to amplify a 353 bp fragment of the glyceraldehyde phosphate dehydrogenase (GAPDH) gene in the *c-KIT* RT reactions. Densitometric analysis of the northern blots was carried out as described in section 4.2.6 below.

4.2.6 Northern blot densitometry

The amount of *c-KIT*, SCF and tubulin mRNAs expressed by cells in various experimental treatment groups was quantified and compared using ImageQuaNT software (Molecular Dynamics) and Microsoft Excel (version 5.0; Microsoft Corp., USA).

Densitometric values were calculated by dividing the hybridization signal intensity of *c-KIT* or SCF mRNA by the corresponding tubulin signal intensity. In some experiments, the treatment group values were expressed as a percent of the control value which was arbitrarily set to 100%.

4.2.7 Culture of HEY cells in the presence of an anti-KIT antibody

HEY cells were seeded at 2×10^4 cells/ml in 500 μ l total volume of α -MEM + 10% FCS, in triplicate wells of a 24-well plate (Nunc Brand Products, Denmark). After 24 h, 1, 5 or 10 μ g/ml of an anti-KIT MAb, SR-1 (generously provided by Dr. L. Bennett, Amgen Inc., Thousand Oaks, CA), was added to the wells. The SR-1 MAb binds to a portion of the extracellular domain of the KIT receptor and neutralizes the binding of SCF to KIT (Broudy *et al.*, 1992). The cells were cultured for an additional 24 h in the presence of the MAb. At the end of the 48 h culture period, the cells were counted using hemacytometry as described in section 2.2.1.

4.2.8 Culture of HEY and OVCA 429 cells in the presence of exogenous SCF

HEY and OVCA 429 cells were seeded at 1.5×10^4 cells/ml in 500 μ l total volume of 10% serum-containing media, in triplicate wells of a 24-well plate. After 24 h, the cells were washed with PBS and the media was replaced with serum-free media containing 0.1, 1, 10, 50 or 100 ng/ml of human SCF (Immunex Corp., Seattle, WA). The SCF was prepared as a 20 μ g/ml sterile stock in PBS containing 0.2% BSA, and control wells received a volume of this diluent equivalent to the highest concentration of SCF. The cells were cultured for

48 h in the presence of SCF, with a second addition of SCF or diluent after the first 24 h. The cells were incubated in the presence of 1 $\mu\text{Ci/ml}$ of [^3H]thymidine for the last 6 or 8 h of the culture period. The cells were then assayed for their DNA incorporation of [^3H]thymidine as a measurement of cellular proliferation as described in section 2.2.2.

4.2.9 Immunocytochemical localization of KIT protein in HEY cells

Immunocytochemical localization and digital imaging of KIT protein in HEY and ACC-LC-80 cells was carried out as described in section 2.5. Additionally, confocal imaging of the immunofluorescently stained cells was performed as follows. Cells were viewed using a rhodamine filter to visualize fluorescence, and were imaged with a Zeiss LSM 410 inverted laser scanning microscope (Carl Zeiss, Thornwood, NY) equipped with an Argon/Krypton ion laser and a Fluar 40X, 1.3 NA oil immersion objective. An electronic zoom of 3.5 was used, which lead to pixels of 0.179 μm , and images were 512 X 512 eight-bit pixels. Optical sections were obtained using the 568 nm excitation wavelengths (bandpass filter 530-585). Emitted fluorescence light was separated at 560 nm using dichroic mirror, and longer wavelengths were filtered through a 590 nm longpass filter prior to detection. Frames of Z-series (usually 20 sections) were taken at regular intervals of 0.5 μm . Optical sections of Z-series were collected as the average of 16 scans to improve the signal-to-noise ratio. Images are displayed as single optical sections (512 X 512) from a Z-series.

4.3 Results

4.3.1 Expression of c-*KIT* and SCF mRNAs and KIT protein in human ovarian carcinoma cells

A panel of 12 human ovarian carcinoma cell lines were screened for expression of c-*KIT* and SCF mRNAs by northern blot analysis in order to identify cells that could be used for functional *in vitro* studies. As shown in Figure 13A, 2 of the 12 ovarian carcinoma lines examined expressed c-*KIT*, HEY and OVCA 429 cells. These 2 cell lines also co-expressed SCF (Figure 13B). Several of the other ovarian carcinoma cell lines expressed SCF but not c-*KIT*: C13*, OCC1, OVCA 420, OVCA 433, OV2008 and SKOV-3.

Western blot analysis to confirm that the c-*KIT* mRNA expressed by HEY and OVCA 429 cells was translated into KIT protein showed that both HEY and OVCA 429 cells expressed detectable KIT protein that was approximately 160 kDa in size (Figure 13D).

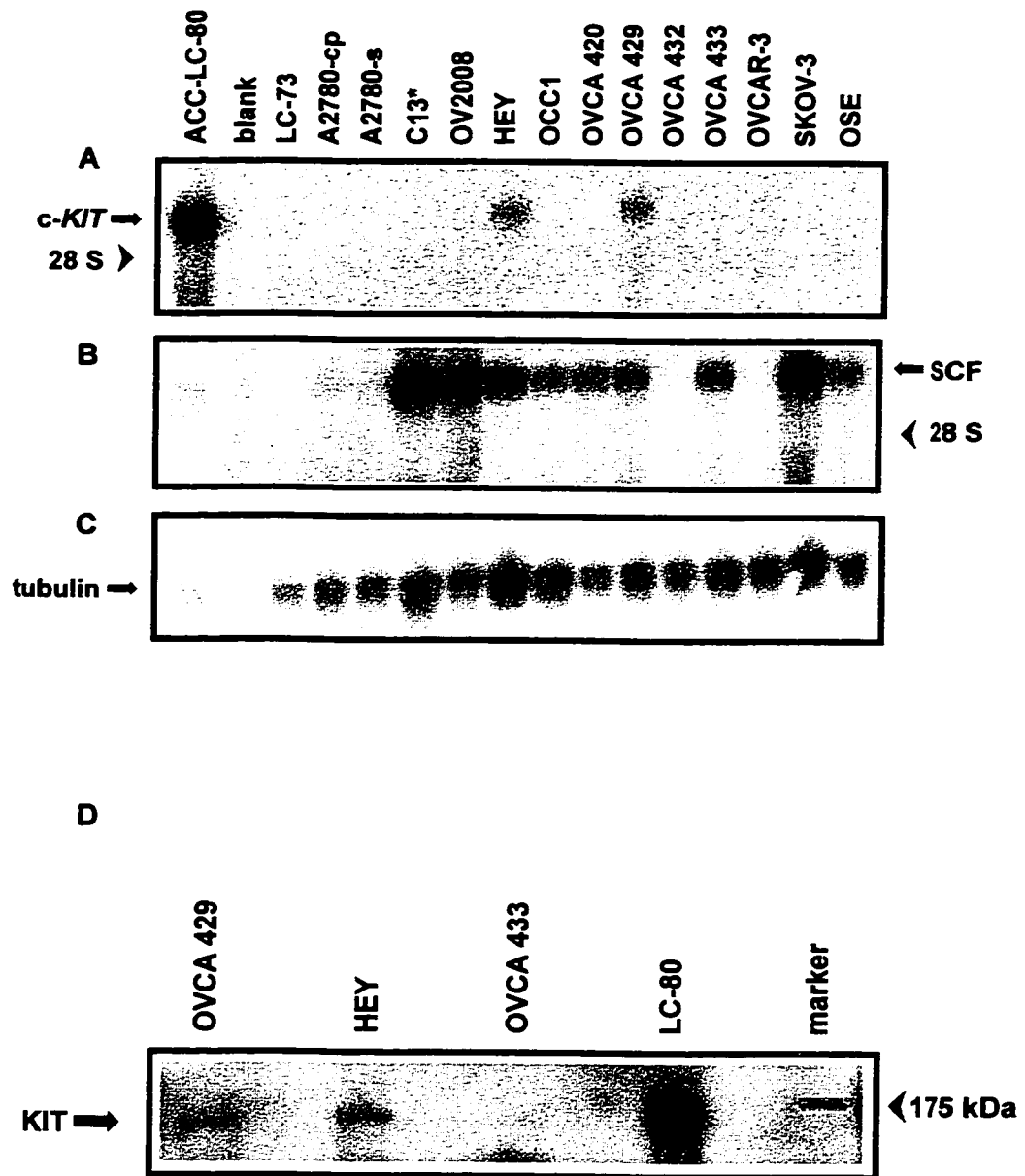
Having determined that HEY and OVCA 429 cells expressed KIT and SCF, a number of *in vitro* studies were carried out to examine factors which could regulate the proliferation and, potentially, the expression of c-*KIT* and SCF in these ovarian carcinoma cells.

4.3.2 Expression of gonadotropin receptors in HEY and OVCA 429 cells

Since gonadotropin regulation of ovarian cancer cell growth has been documented, the expression of LH-R and FSH-R mRNAs was analyzed in HEY and OVCA 429 cells using northern blot analysis. Both LH-R and FSH-R genes encode for multiple mRNA transcripts that are detectable by northern blot analysis (McFarland *et al.*, 1989; Dunkel *et*

Figure 13

Expression of *c-KIT* and SCF in human ovarian carcinoma cells. Expression of *c-KIT* and SCF mRNAs was determined by northern blot analysis using 15 µg of total RNA extracted from cultured cells, and sequential probing with a [³²P]-labeled human *c-KIT* cDNA (A), human SCF cDNA (B), and tubulin cDNA (to indicate levels of RNA loaded; C). ACC-LC-80 small cell lung cancer cells were used as the positive control for *c-KIT* (only 5 µg of RNA was loaded since these cells over-express *c-KIT*) and SCF mRNA expression, while LC-73 squamous cell lung cancer cells served as the negative control for both *c-KIT* and SCF expression. Two of the twelve ovarian carcinoma cell lines examined expressed the 5.1 kb *c-KIT* mRNA, HEY and OVCA 429 cells, and both co-expressed the 6.0 kb SCF mRNA. Confirmation that HEY and OVCA 429 cells expressed KIT protein was done by western blot analysis (D) of total protein lysates using a mouse anti-human KIT MAb. ACC-LC-80 cells were used as the positive control for KIT protein expression, while OVCA 433 ovarian carcinoma cells served as the negative control. Both HEY and OVCA 429 cells expressed detectable levels of the 160 kD KIT protein.



al., 1994; Zhang *et al.*, 1994). Using rat cDNA probes, HEY and OVCA 429 cells were found to express a 2.7 kb LH-R transcript and HEY cells also expressed a 4.2 kb LH-R transcript (Figure 14A). When examined for FSH-R expression, both HEY and OVCA 429 cells expressed a 4.2 kb transcript (Figure 14B). In contrast to the ovarian carcinoma cells, neither primary rat OSE cells nor ROSE 199 cells expressed either LH-R (Figure 14A) or FSH-R (Figure 14B). Despite the presence of LH-R and FSH-R transcripts in HEY and OVCA 429 cells, there was no change in either the proliferation or the expression of *c-KIT* and SCF in these cells in response to treatment with 120 ng/ml FSH or 5 IU/ml hCG (Keszthelyi *et al.*, submitted).

Binding of the gonadotropins to their cell surface receptors activates adenylyl cyclase which causes intracellular cAMP accumulation, activation of cAMP-dependent protein kinases and, ultimately, gene activation. Since loss of gonadotropin receptor expression has been documented in cultured cells (Fitz *et al.*, 1989), to circumvent the possibility that the HEY and OVCA 429 cells did not express functional gonadotropin receptors, activation of the FSH-R and LH-R was mimicked using dbcAMP in subsequent experiments.

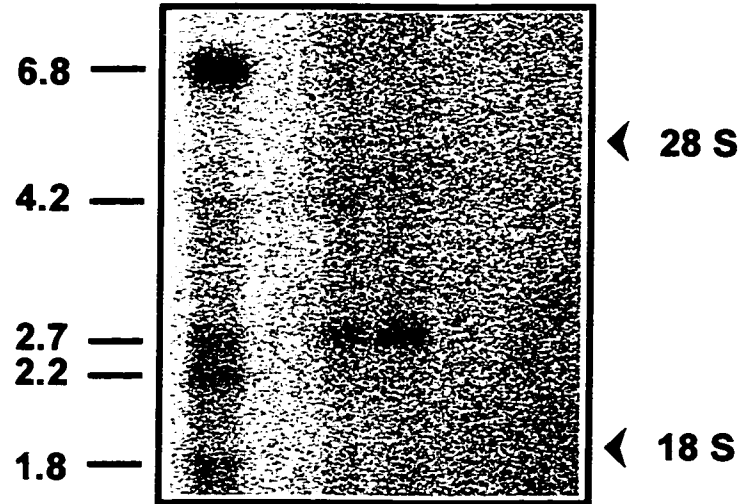
4.3.3 Regulation of HEY cell proliferation by dbcAMP and growth factors

Determination of the effects of dbcAMP and growth factors on proliferation and *c-KIT* and SCF expression was carried out using HEY cells. Figure 15 demonstrates how the proliferation of HEY cells was affected by culture in the presence of dbcAMP and growth factors, either alone or in combination. HEY cells could be stimulated to proliferate significantly more rapidly when cultured in the presence of TGF- α ($p < 0.001$), but their

Figure 14

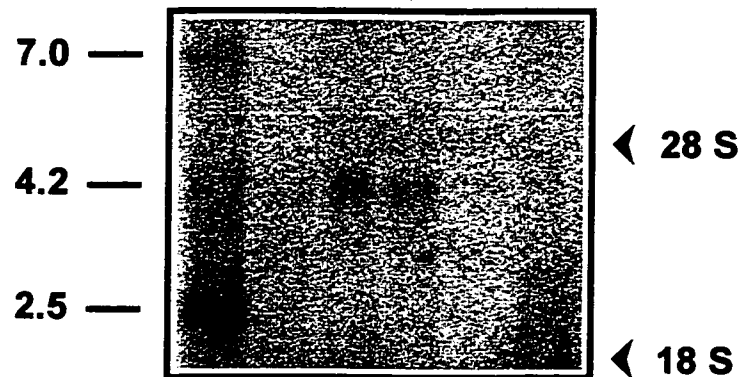
Expression of gonadotropin receptors in HEY and OVCA 429 cells but not in rat OSE cells. Determination of the expression of LH-R and FSH-R in HEY, OVCA 429, primary rat OSE cells and ROSE 199 cells was done using northern blot analysis. Total RNA (15 µg) was consecutively probed with [³²P]-labeled cDNAs for rat LH-R (A), rat FSH-R (B), and tubulin (C). Cultured rat granulosa cells (gc) harvested 6 h after hCG treatment (Ismail *et al.*, 1996) were used as the positive control for LH-R and FSH-R expression, while NIH 3T3 fibroblast cells served as the negative control. HEY and OVCA 429 cells expressed a 2.7 kb LH-R transcript and HEY cells also expressed a 4.2 kb LH-R transcript. Both cell types expressed a 4.2 kb FSH-R transcript. In contrast to the ovarian carcinoma cell lines, normal rat OSE cells and ROSE 199 cells did not express either LH-R or FSH-R.

A LH-R



rat gc
NIH 3T3
HEY
OVCA 429
rat OSE
ROSE 199

B FSH-R



C

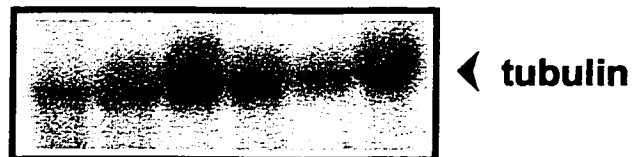
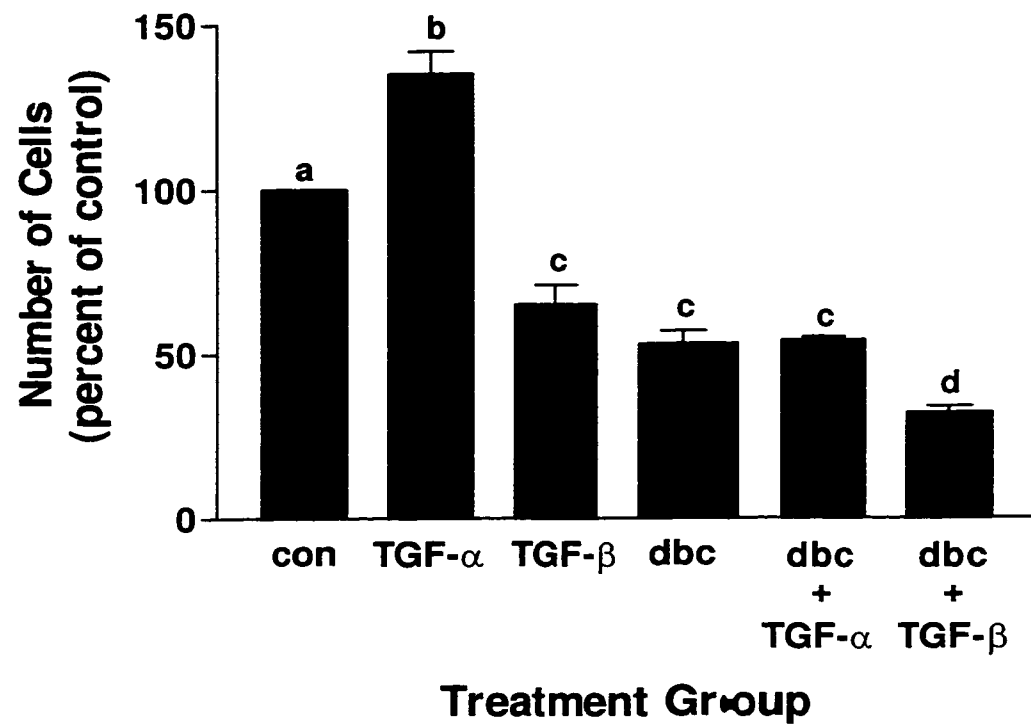


Figure 15

Proliferation of HEY ovarian carcinoma cells is regulated by dibutyryl cAMP and growth factors. HEY cells were cultured for 48 h in the absence (con) or presence of 2 mM dibutyryl cAMP (dbc), 10 ng/ml transforming growth factor- α (TGF- α), or 10 ng/ml transforming growth factor- β_1 (TGF- β), either alone or in combination. Proliferation was assessed at the end of the culture period by counting the cells using hemacytometry. Bars represent the mean $\pm$ SEM of at least 9 separate values from 3-5 experiments, and are expressed as a percentage of the control mean which was arbitrarily set to 100%. Different lowercase letters above the bars indicate significant differences in the number of cells in those treatment groups. HEY cells proliferated significantly more rapidly when cultured in the presence of TGF- α ($p < 0.001$), while their proliferation was inhibited equally by dbc ($p < 0.001$) or TGF- β ($p < 0.001$). The inhibitory effect of dbc predominated when HEY cells were cultured in the presence of both dbc and TGF- α , such that the proliferation of the cells was decreased to the same extent as was observed with dbc alone. When dbc and TGF- β were given in combination to the HEY cells, there was a greater inhibitory effect on proliferation than was seen with either agent alone ($p < 0.05$).



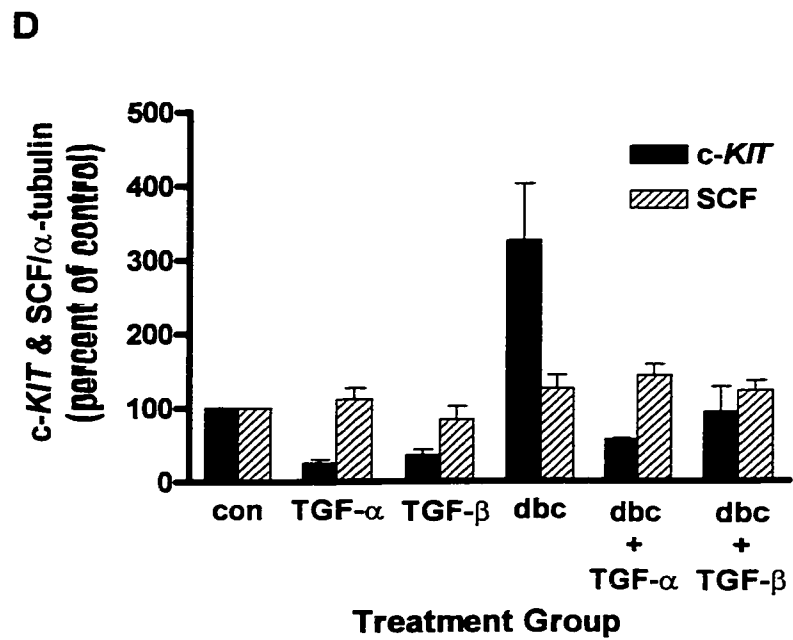
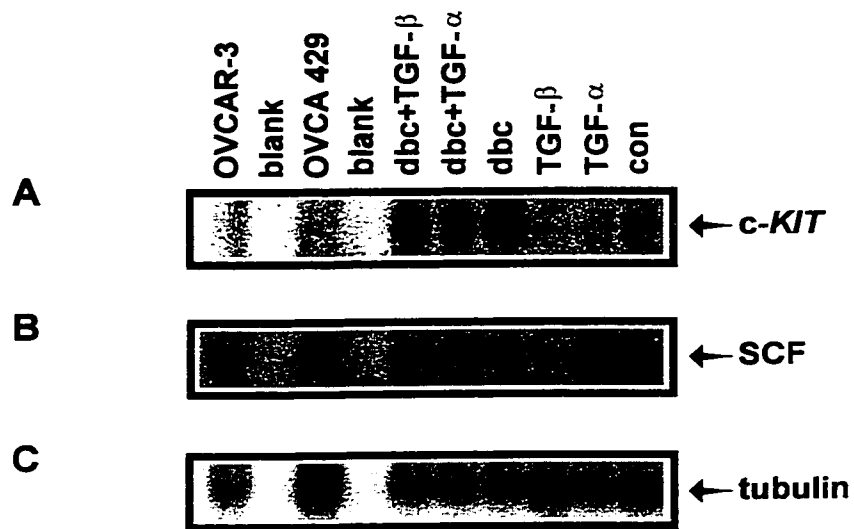
proliferation was inhibited in the presence of both dbcAMP ($p<0.001$) and TGF- β ($p<0.001$). The growth inhibition caused by dbcAMP and TGF- β was equivalent at the concentrations used, with both agents causing a 40-50% decrease in HEY cell proliferation relative to the control, untreated cells. The inhibitory effect of the dbcAMP predominated when HEY cells were cultured in the presence of dbcAMP and TGF- α concurrently, since the stimulatory effect on proliferation that was observed when HEY cells were exposed to TGF- α alone was abolished. When dbcAMP and TGF- β were given in combination to the HEY cells, there was a greater inhibitory effect on proliferation than was seen with either agent alone, such that proliferation was reduced to 30% of that seen with the control, untreated cells. There was no evidence of cell death, i.e., increased numbers of floating cells, in cultures of HEY cells exposed to dbcAMP or TGF- β .

4.3.4 Regulation of *c-KIT* but not SCF mRNA expression in HEY cells by dbcAMP and growth factors

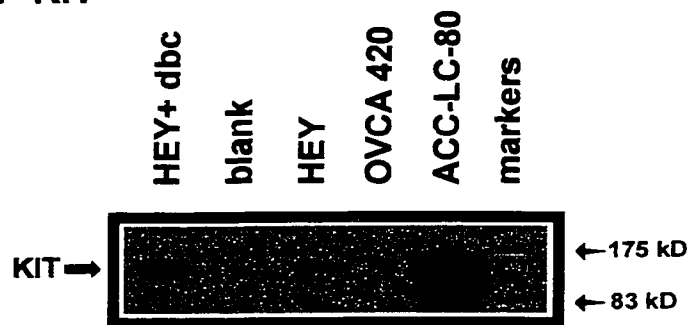
In addition to regulating HEY cell proliferation, dbcAMP and the growth factors also influenced *c-KIT* expression in these cells. Dibutyl cAMP treatment of HEY cells resulted in an approximate 3-fold upregulation of *c-KIT* mRNA expression (Figure 16A, D). The dbcAMP-associated increase in *c-KIT* mRNA expression was also evident at the KIT protein level (Figure 16E). Treatment of HEY cells with either TGF- α or TGF- β caused a decrease in *c-KIT* expression relative to control, untreated cells (Figure 16A, D). Interestingly, the dbcAMP-associated increase in HEY cell *c-KIT* expression was not observed if the cells were cultured with dbcAMP concurrently with either TGF- α or TGF- β ; instead, *c-KIT*

Figure 16

Expression of c-*KIT* and SCF in HEY ovarian carcinoma cells is regulated by dibutyryl cAMP and growth factors. HEY cells were cultured for 48 h in the absence (con) or presence of 2 mM dibutyryl cAMP (dbc), 10 ng/ml transforming growth factor- α (TGF- α), or 10 ng/ml transforming growth factor- β_1 (TGF- β), either alone or in combination. Expression of c-*KIT* and SCF mRNAs was determined by northern blot analysis using 15 μ g of total RNA extracted from the cells, and sequential probing with a [32 P]-labeled human c-*KIT* cDNA (A), human SCF cDNA (B), and tubulin cDNA (to indicate levels of RNA loaded; C). OVCA 429 ovarian carcinoma cells served as the positive control for c-*KIT* and SCF mRNA expression, while OVCAR-3 ovarian carcinoma cells were used as the negative control for both c-*KIT* and SCF. Bars in the histogram (D) represent the relative amount of c-*KIT* and SCF mRNA expressed by HEY cells in each treatment group, expressed as a percentage of the c-*KIT*:tubulin or SCF:tubulin in the control, untreated cells. Data in A-C is a representative blot from one of two independent experiments, while results in D show the mean from both experiments' blots and the error bars represent the minimum and maximum values. In panel E, HEY cells were cultured for 24 h in the absence (HEY) or presence of 2 mM dbc (HEY + dbc) and then western blot analysis of immunoprecipitated KIT protein was performed. Culture of HEY cells in the presence of TGF- α or TGF- β caused an approximate 70% decrease in c-*KIT* mRNA expression, while treatment with dbc resulted in an approximate 3-fold increase in c-*KIT* expression which was also evident at the protein level. Exposure of HEY cells to dbc concurrently with either growth factor prevented the dbc-induced increase in c-*KIT* expression. The expression of SCF in HEY cells did not change relative to control, untreated cells when they were cultured in the presence of dbc or growth factors, either alone or in combination.



E IP KIT



expression was suppressed to a level between control, untreated cells and cells treated with TGF- α or TGF- β alone (Figure 16A, D). In contrast to the changes in *c-KIT* mRNA expression, SCF expression was not affected by dbcAMP and/or the growth factors (Figure 16B, D).

RT-PCR analysis to determine whether dbcAMP or the growth factors altered the proportion of membrane-associated versus soluble *c-KIT* and SCF that was expressed by HEY cells revealed that there was no change in the predominant expression of membrane-associated *c-KIT* and soluble SCF (data not shown).

4.3.5 Expression of *c-KIT* and SCF in HEY and OVCA 429 cells at various confluencies in culture

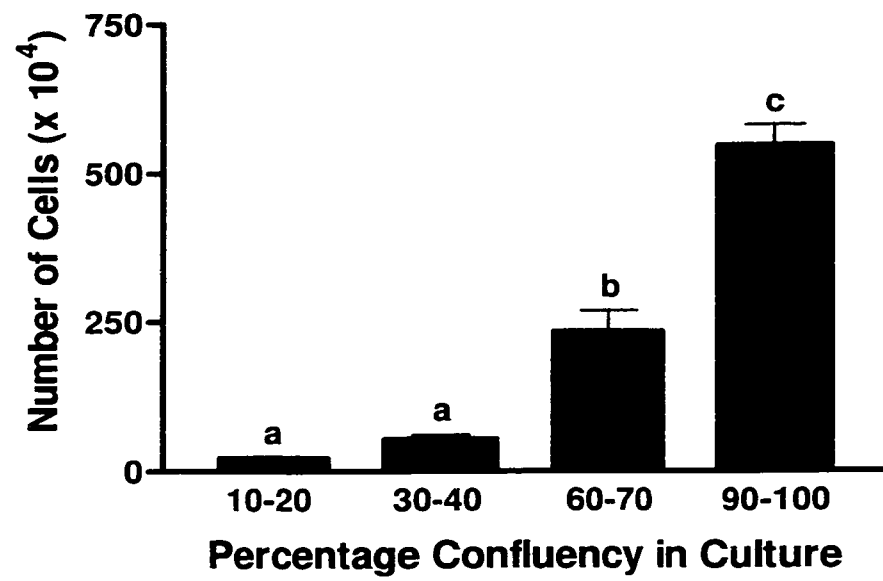
To assess whether cell confluency impacted on *c-KIT* and SCF expression, HEY and OVCA 429 cells were cultured in their regular maintenance medium (Table 1) for a total of 96 h and their proliferation and level of *c-KIT* and SCF mRNA expression were quantified at 24 h intervals. The cells were 10-20% confluent after 24 h of culture, 30-40% confluent after 48 h, 60-70% confluent after 72 h, and following 96 h of culture the cells reached 90-100% confluence. The actual cell numbers corresponding to these cell confluencies for both the HEY and OVCA 429 cells are shown in Figure 17.

Analysis of the expression of *c-KIT* and SCF in HEY and OVCA 429 cells at the various confluencies revealed some interesting patterns. HEY cell *c-KIT* mRNA expression declined as the cell density increased; in fact, *c-KIT* expression was not detectable in cells at the two highest cell confluencies when examined by northern blot analysis (Figure 18A,

Figure 17

The number of HEY and OVCA 429 cells corresponding to various confluencies in culture. HEY and OVCA 429 cells were seeded at 1×10^5 or 1.5×10^5 cells, respectively, and their cell numbers were determined 24, 48, 72, and 96 h later using hemacytometry. These time points corresponded to cell confluencies of 10-20%, 30-40%, 60-70% and 90-100%, respectively. Bars represent the mean $\pm$ SEM of 9 separate values from 3 experiments. Different lowercase letters above the bars indicate significant differences in the number of cells at those confluencies in culture.

(A) HEY



(B) OVCA 429

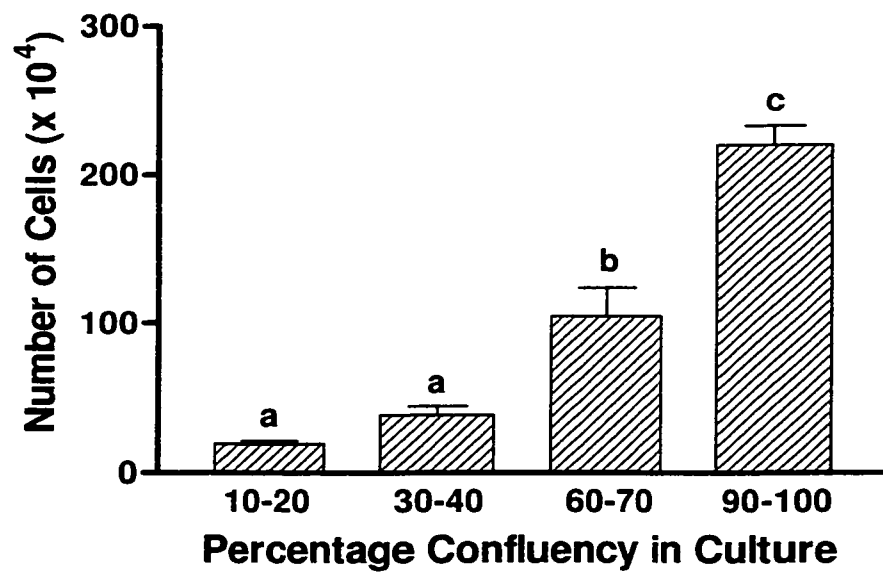
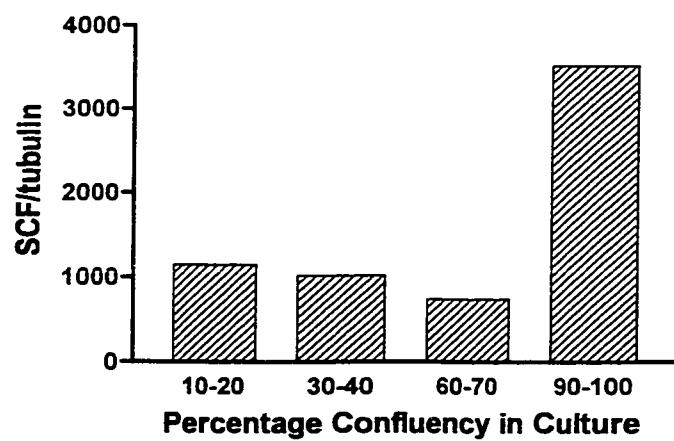
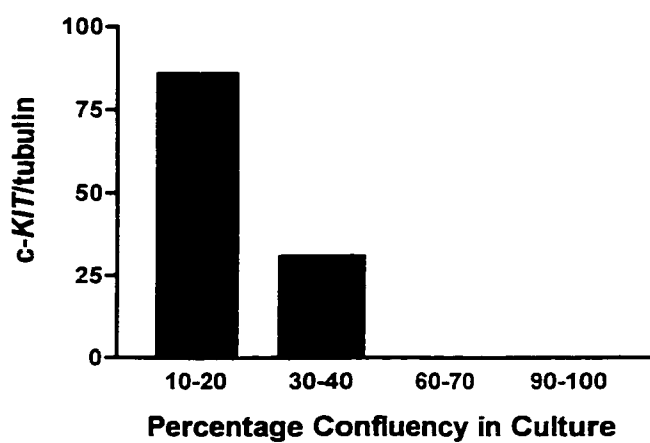
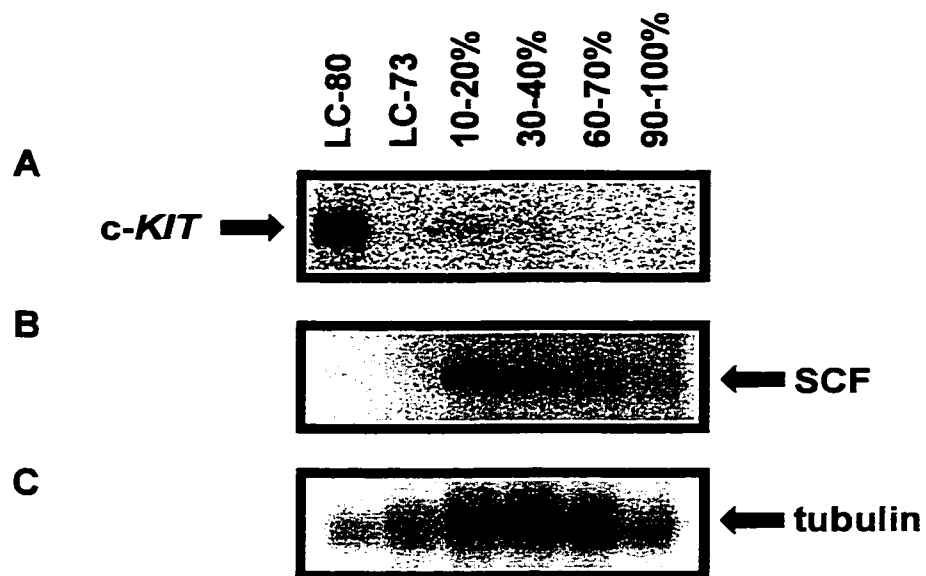


Figure 18

Expression of c-*KIT* and SCF mRNAs in HEY ovarian carcinoma cells is regulated by their confluency in culture. Expression of c-*KIT* and SCF mRNAs in HEY cells grown to confluencies in culture of 10-20, 30-40, 60-70, and 90-100% was determined by northern blot analysis using 15 µg of total RNA extracted from the cells, and sequential probing with a [³²P]-labeled human c-*KIT* cDNA (A), human SCF cDNA (B), and tubulin cDNA (to indicate levels of RNA loaded; C). ACC-LC-80 small cell lung cancer cells were used as the positive control for c-*KIT* (only 5 µg of RNA was loaded since these cells over-express c-*KIT*) and SCF mRNA expression, while LC-73 squamous lung cancer cells served as the negative control for both c-*KIT* and SCF expression. Bars in the histograms (D, E) represent the relative amount of c-*KIT* and SCF mRNA expressed by HEY cells at each confluency, expressed as the ratio of the amount of c-*KIT* or SCF relative to the respective tubulin internal control. Data in A-E are a representative blot with densitometric analysis from one of two independent experiments which showed similar results. C-*KIT* expression decreased in HEY cells as their level of confluency in culture increased, with no detectable c-*KIT* mRNA at the higher cell confluencies (60-100%). HEY cell SCF mRNA expression was unchanged at confluencies ranging from 10-70%, but was increased at higher confluencies.



D). RT-PCR analysis confirmed that HEY cells recovered from the most highly confluent dishes did not express *c-KIT* despite abundant expression of a housekeeping gene, GAPDH (Figure 19A, B). Furthermore, despite changes in the overall level of *c-KIT* expression, there was no change in the relative abundance of the two isoforms encoding *c-KIT*, with the 591 bp membrane-associated isoform always present in greater abundance (Figure 19A). In contrast to *c-KIT* expression, SCF expression was unchanged in HEY cells at densities ranging from 10% to 70%, but was increased at higher confluencies (Figure 18B,E). There was no change in the predominant expression of the 287 bp soluble isoform of SCF relative to the 203 bp membrane-associated isoform (Figure 19C).

The expression of *c-KIT* and SCF in the OVCA 429 cells at various cell confluencies differed somewhat from that observed in HEY cells. Specifically, expression of *c-KIT* in OVCA 429 did not change as the cell confluency was increased (Figure 20A, D). However, OVCA 429 expression of SCF progressively increased in parallel with increased cell confluency (Figure 20B, D). RT-PCR analysis of the relative expression of *c-KIT* and SCF isoforms revealed no change in the predominant expression of membrane-associated *c-KIT* and soluble SCF in the OVCA 429 cells at the different cell confluencies (data not shown).

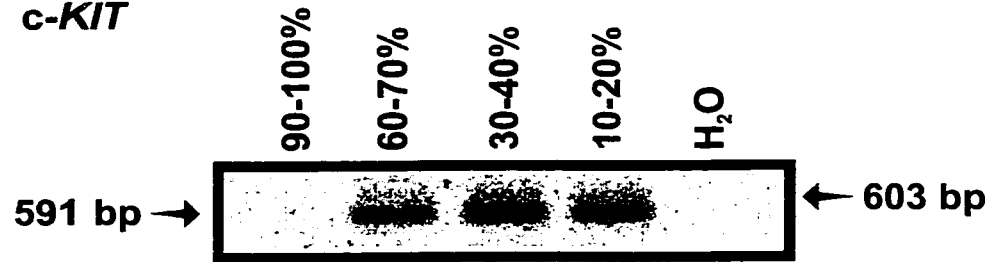
4.3.6 Proliferation of HEY or OVCA 429 cells in the presence of an anti-KIT neutralizing antibody or exogenous SCF

Having determined that HEY and OVCA 429 cells expressed *c-KIT* mRNA and KIT protein, experiments were carried out to assess a potential proliferative function of the KIT:SCF interaction in these cells. HEY cells were cultured in the presence of a neutralizing

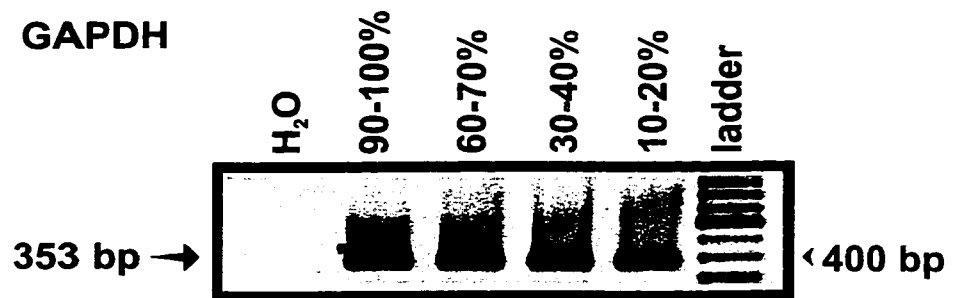
Figure 19

Reverse transcription-polymerase chain reaction analysis of c-*KIT* and SCF isoforms in HEY cells at various confluencies in culture. RT-PCR was performed on total RNA isolated from HEY cells grown to cell confluencies in culture of 10-20, 30-40, 60-70, and 90-100%. C-*KIT* primers (A) were used to amplify the 603 bp and 591 bp fragments representative of the soluble and membrane-associated isoforms, respectively, of the KIT receptor. As an internal control, glyceraldehyde phosphate dehydrogenase (GAPDH) primers (B) were used to amplify a 353 bp fragment in the c-*KIT* RT reactions. SCF primers (C) were used to amplify the 287 bp and 203 bp fragments representative of the soluble and membrane-associated isoforms, respectively, of SCF. For HEY cell confluencies ranging from 10-70%, the 591 bp membrane-associated c-*KIT* isoform was always predominant. HEY cell c-*KIT* expression was not detectable in cells at 90-100% confluency in culture despite abundant GAPDH expression. At all confluencies, HEY cells expressed relatively more of the 287 bp soluble SCF isoform compared to the 203 bp membrane-associated isoform.

(A) c-KIT



(B) GAPDH



(C) SCF

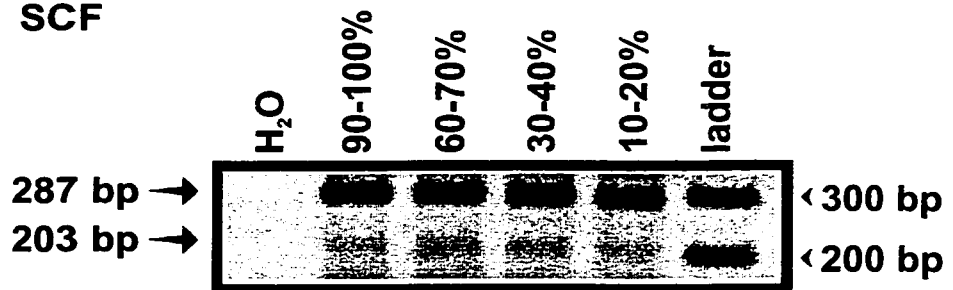
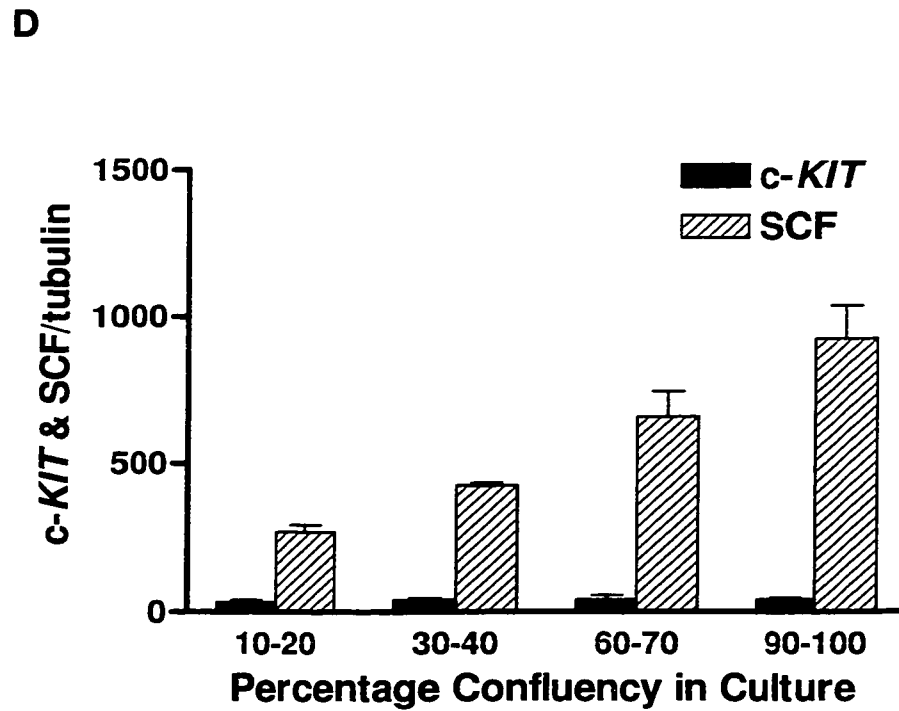
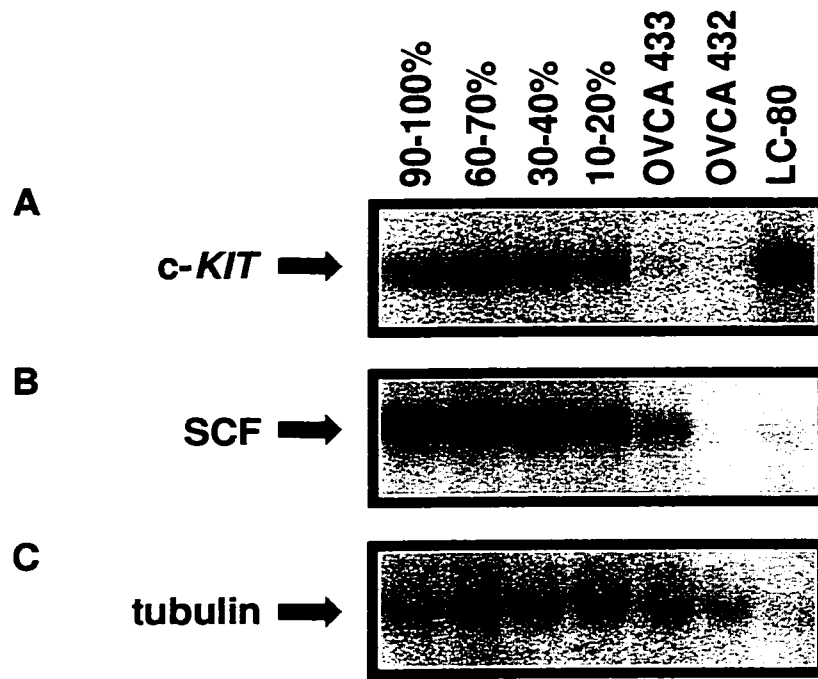


Figure 20

Expression of SCF, but not *c-KIT*, by OVCA 429 cells is regulated by confluency in culture. Expression of *c-KIT* and SCF mRNAs in OVCA 429 cells grown to confluencies in culture of 10-20, 30-40, 60-70, and 90-100% was determined by northern blot analysis using 15 µg of total RNA extracted from the cells, and sequential probing with a [³²P]-labeled human *c-KIT* cDNA (A), human SCF cDNA (B), and tubulin cDNA (to indicate levels of RNA loaded; C). ACC-LC-80 small cell lung cancer cells were used as the positive control for *c-KIT* expression (only 5 µg of RNA was loaded since these cells over-express *c-KIT*). OVCA 433 ovarian carcinoma cells served as the positive control for SCF mRNA expression, and OVCA 432 ovarian carcinoma cells were used as the negative control for both *c-KIT* and SCF. Bars in the histogram (D) represent the relative amount of *c-KIT* and SCF mRNA expressed by OVCA 429 cells at each confluency, expressed as the ratio of the amount of *c-KIT* or SCF relative to the respective tubulin internal control. Data in A-C is a representative blot from one of two independent experiments, while results in D show the mean from both experiments' blots and the error bars represent the minimum and maximum values. *C-KIT* expression remained unchanged in OVCA 429 cells regardless of their confluency in culture, while SCF expression progressively increased in these cells as their confluency increased.



anti-KIT MAb, SR-1, to block SCF-mediated activation of the KIT receptor and cellular proliferation was assessed. Figure 21A shows that the proliferation of HEY cells was unaffected by the SR-1 MAb at concentrations up to 10 µg/ml.

In order to assess whether further stimulation of the KIT receptor by exogenous SCF could affect the proliferation of OVCA 429 and HEY cells, the cells were cultured in the presence of 0.1-100 ng/ml of SCF in serum-free media. The use of serum-free media ensured that any changes in the proliferation of the cells could be attributed to SCF and not to other growth-regulating factors present in serum (including SCF). However, as shown in Figure 21B, the proliferation of OVCA 429 cells was not influenced by any concentration of SCF. Indeed, the proliferation of OVCA 429 cells was not affected by concentrations of SCF as high as 500 ng/ml (data not shown). Proliferation of HEY cells was also unaffected by SCF at concentrations of 0.1-500 ng/ml of SCF (data not shown).

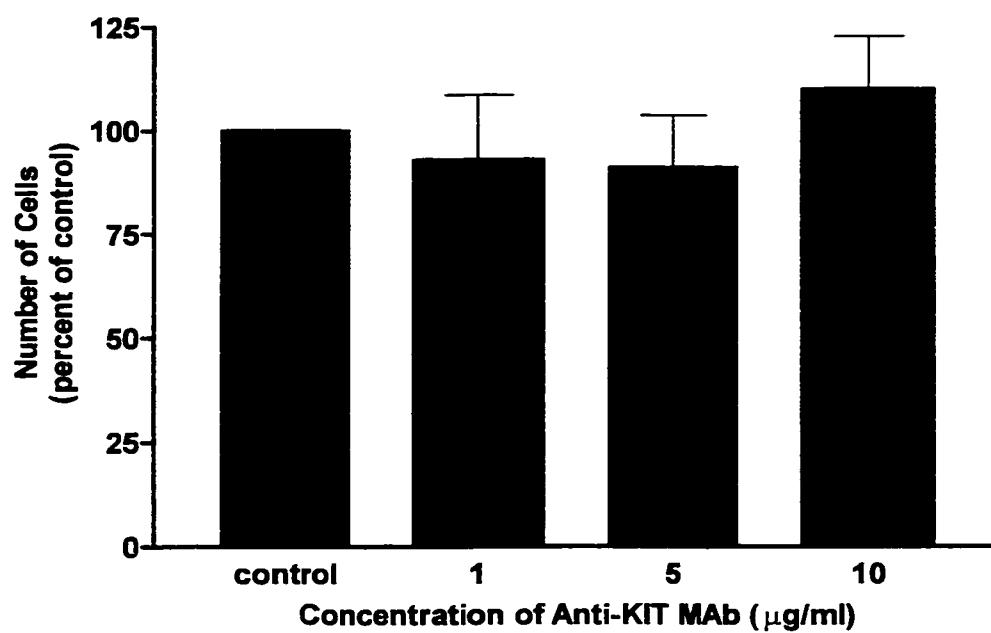
4.3.3 Subcellular localization of KIT protein in HEY cells

In an effort to understand why blocking activation of the KIT receptor in HEY cells using a neutralizing anti-KIT MAb did not affect the proliferation of the cells, the subcellular localization of KIT protein in HEY cells was assessed. Immunocytochemical analysis of KIT protein expression in HEY cells using both fluorescence (Figure 22e) and confocal microscopy (Figure 22f) revealed that KIT was not found at the HEY cell surface, but rather was expressed in the cytoplasm of the cells, with a distinct peri-nuclear intensity of staining (Figure 22f). In contrast to the HEY cells, ACC-LC-80 lung cancer cells, which were used

Figure 21

Proliferation of HEY or OVCA 429 ovarian carcinoma cells is unaffected by a neutralizing anti-KIT antibody or exogenous SCF. HEY cells were cultured for the second 24 h of a 48 h culture period in the absence (control) or presence of 1, 5, or 10 $\mu\text{g/ml}$ of SR-1, a neutralizing anti-KIT MAb (A), and the number of cells at the end of the culture time was determined using hemacytometry. OVCA 429 cells were cultured for the first 24 h of a 72 h culture period in serum-containing media, and for the next 48 h in serum-free media lacking (control) or containing 0.1-100 ng/ml of human SCF (B). During the last 6 or 8 h of culture, the OVCA 429 cells were incubated with 1 $\mu\text{Ci/ml}$ of [^3H]thymidine, after which time they were assayed for the amount of [^3H]thymidine incorporated into DNA. Bars represent the mean $\pm$ SEM of at least 9 separate values from 3-4 experiments and are expressed as a percent of the control value which was arbitrarily set to 100%. The proliferation of HEY cells was not affected by culture with SR-1, and proliferation of OVCA 429 cells was not influenced by treatment with exogenous SCF.

(A) HEY



(B) OVCA 429

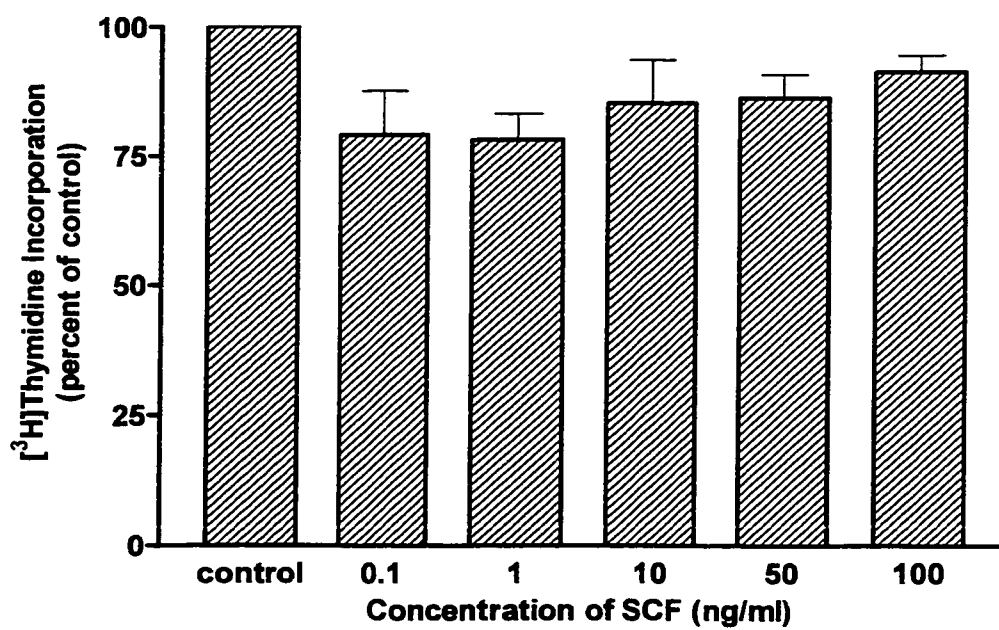
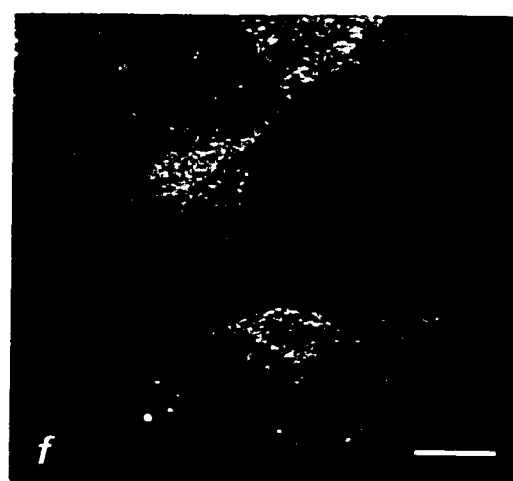
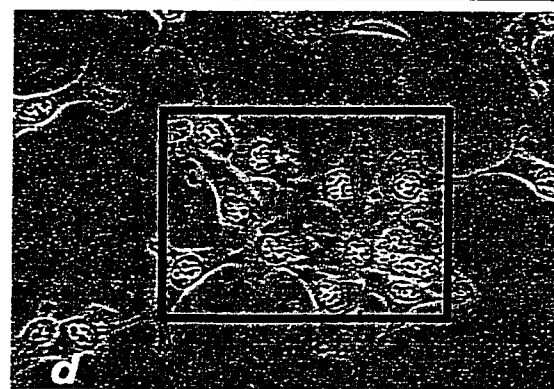
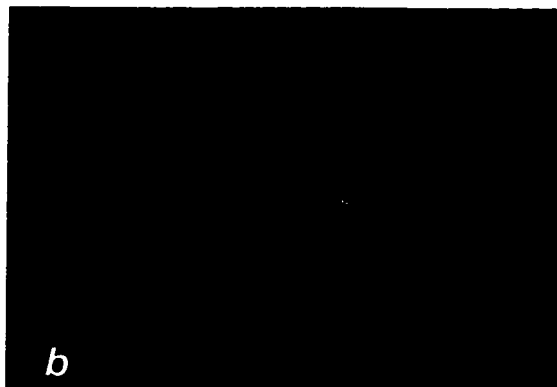
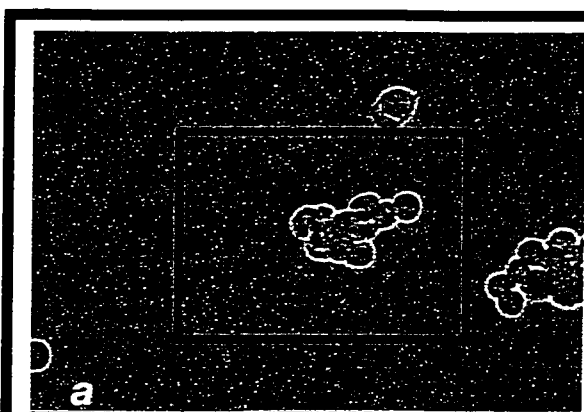


Figure 22

Immunocytochemical detection of KIT protein in HEY ovarian carcinoma cells. HEY cells were grown on coverslips and stained for KIT protein using a rabbit anti-human KIT polyclonal primary antibody and a CYTM3-conjugated secondary IgG. ACC-LC-80 small cell lung cancer cells served as the positive control for KIT protein expression. Panels *a-c* are the ACC-LC-80 cells and panels *d-f* are the HEY cells. Cells are shown in phase-contrast (*a, d*), with immunofluorescence (*b, e*), and with confocal imaging of immunofluorescence (*c, f*). Phase-contrast images are magnified 200X, while the fluorescent images are magnified 400X and correspond to the boxed areas of their phase-contrast images. The confocal images are of ACC-LC-80 and HEY cells from a different experiment than those shown in the top panels (scale bar = 10 μ M). In contrast to the ACC-LC-80 cells which showed clear membrane-associated KIT protein expression, HEY cell KIT protein was detectable as a diffuse cytoplasmic expression with a distinct peri-nuclear localization.

ACC-LC-80 lung cancer cells

HEY ovarian cancer cells



as the positive control in the immunocytochemistry procedure, showed clear membrane-associated staining of KIT protein particularly at regions of juxtaposed cell membranes (Figure 22*b, c*). Notably, OVCA 429 cells showed the identical localization of KIT protein as was observed in the HEY cells (data not shown).

4.4 Discussion

The screening of a panel of twelve ovarian carcinoma cell lines for c-*KIT* and SCF expression revealed that two of the twelve lines, HEY and OVCA 429 cells, expressed both c-*KIT* and SCF. The steady-state levels of c-*KIT* and/or SCF expression in these cells could be differentially regulated by dbcAMP, growth factors, and confluency in culture. Although both HEY and OVCA 429 cells expressed KIT protein, it was localized in the cytoplasm and not at the plasma membrane, and their proliferation was unaffected by culture in the presence of a neutralizing anti-KIT antibody or exogenous SCF.

In contrast to the greater than 70% of epithelial ovarian tumors that expressed c-*KIT* (Chapter 3), only two of twelve ovarian carcinoma cell lines (17%) expressed KIT. It is possible that the lack of c-*KIT* expression in the other ovarian carcinoma lines may reflect a loss of KIT receptor expression during or following the immortalization of those cells *in vitro*. For example, loss of EGF-R and LH-R expression has been reported in SV40-immortalized rat granulosa cells in culture (Fitz *et al.*, 1989). Such downregulated expression may be due to a loss of microenvironmental factors that promote c-*KIT* expression *in vivo*, or it may be a consequence of molecular changes required for growth regulation during the prolonged maintenance of the cells in culture. Given that loss of c-*KIT* expression in

epithelial ovarian tumors appears to correlate with a more aggressive disease state, it is perhaps a deliberate mechanism on the part of the ovarian carcinoma cells to shut off *c-KIT* expression (perhaps via gene silencing) to promote their survival in culture. Cultured human (Chapter 3) and rat (Ismail *et al.*, 1999) OSE cells clearly expressed SCF, and this expression was maintained in all but two (OVCA 432 and OVCAR-3) of the ovarian carcinoma cell lines.

In this study, exogenous TGF- α significantly stimulated the proliferation of HEY ovarian carcinoma cells compared to untreated control cells. In itself, this effect of TGF- α on HEY cell proliferation was not a novel finding, since Jindal *et al.* (1994) reported that HEY cells expressed TGF- α mRNA and secreted TGF- α peptide, and that the cells exhibited a 2-fold increase in proliferation when treated with exogenous TGF- α in serum-free culture. However, TGF- α treatment of HEY cells also resulted in downregulation of *c-KIT* mRNA expression in HEY cells in this study. This is the first report of TGF- α regulation of *c-KIT* in any cell type. Whether there is a functional link between the roles of TGF- α to stimulate HEY cell proliferation and decrease *c-KIT* expression is discussed below.

Exposure of HEY cells to exogenous TGF- β resulted in a significant decrease in the proliferation of these cells compared to untreated control cells. Although HEY cells had been previously shown to express TGF- β mRNA and immunoreactive TGF- β protein, as well as mRNA for the type II, betaglycan and endoglin TGF- β receptors (Jindal *et al.*, 1995), the effect of exogenous TGF- β on the growth of HEY cells has not been reported and, therefore, this represents a novel finding in this study. However, it is not surprising to find that TGF- β can decrease the proliferation of HEY cells since normal human OSE cells in primary culture

as well as many ovarian cancer cells have been shown to be growth-inhibited by TGF- β (Berchuck *et al.*, 1992; Hurteau *et al.*, 1994; Havrilesky *et al.*, 1995).

In addition to affecting the proliferation of HEY cells, TGF- β treatment also caused an approximate 70% decrease in c-*KIT* mRNA expression in these cells without altering SCF expression. While this is the first demonstration of TGF- β regulating c-*KIT* expression in human ovarian cancer cells, this finding is in agreement with other reports of TGF- β -induced downregulation of c-*KIT* mRNA and/or protein expression in human hematopoietic progenitor cells (Dubois *et al.*, 1994; Heinrich *et al.*, 1995; Sansilvestri *et al.*, 1995), leukemic myeloblasts (Heinrich *et al.*, 1995), and HT-29 colorectal cancer cells (Bellone *et al.*, 1997). Although the mechanism by which TGF- β downregulated c-*KIT* expression in HEY cells is not known at this time, in other cell types this repression has been associated with accelerated degradation of the c-*KIT* mRNA transcripts (de Vos *et al.*, 1993; Dubois *et al.*, 1994; Heinrich *et al.*, 1995).

Despite the presence of LH-R and FSH-R mRNA transcripts in HEY and OVCA-429 cells, it was unclear whether these cells produced functional gonadotropin receptors since they had been previously shown to be unresponsive to exogenous treatment with hCG or FSH (Keszthelyi *et al.*, submitted). Both gonadotropin receptors are known to use cAMP as an immediate downstream effector molecule following ligand-receptor binding, so activation of this pathway was mimicked using the membrane-permeable cAMP analogue, dbcAMP. Treatment of HEY cells with dbcAMP caused a significant inhibition of cell proliferation in a 48 h period. Treatment of HEY cells with two other agents that induce accumulation of cAMP, 8-bromo-cAMP or cholera toxin, also caused decreased proliferation of HEY cells.

(Keszthelyi *et al.*, submitted). Growth inhibitory effects mediated by cAMP analogues have been well documented for a variety of cell types, including those of tumor origin (Laskin *et al.*, 1990; Mohan and Baylink, 1991; Ohmura *et al.*, 1993; Kamiya *et al.*, 1995; Huttner *et al.*, 1996). The results presented here suggest that ovarian cancer cells resemble other cancer cell types in demonstrating cAMP-induced suppression of proliferation.

In contrast to HEY and OVCA 429 cells, normal rat OSE cells and ROSE 199 cells did not express LH-R and FSH-R mRNAs. However, gonadotropin receptor expression and gonadotropin responsiveness have been reported for human and rabbit OSE cells (Osterholzer *et al.*, 1985b; Zheng *et al.*, 1996; Konishi *et al.*, 1999), suggesting that species-specific differences may exist. Alternatively, since an LH-stimulated increase in P_4 production was previously documented in ROSE 199 cells (R. Ismail, Ph.D. thesis, 1997), perhaps the amount of LH-R expression in these cells was simply below the level of detection of northern blot analysis.

In addition to the effect on cellular proliferation, activation of the cAMP signaling pathway increased the expression of *c-KIT* mRNA and KIT protein in HEY cells. Similar cAMP-mediated upregulation of *c-KIT* has been reported in human erythroleukemia cells (Ogawa *et al.*, 1995) and in differentiated F9 teratocarcinoma cells (Nishina *et al.*, 1992), and has been associated with an increased half-life of the *c-KIT* mRNA (Ogawa *et al.*, 1995). It is unlikely that cAMP-mediated upregulation of *c-KIT* mRNA expression in cells is a direct effect of this second messenger, since there is no cAMP response element in the *c-KIT* gene promoter. However, *c-KIT* transcription might be regulated *in trans* by a transcription factor whose expression or activation is induced by cAMP. Whether a functional link exists

between cAMP-mediated inhibition of proliferation and increased *c-KIT* expression is discussed below.

The expression of *c-KIT* in HEY cells was also modulated by their confluency in culture, i.e., *c-KIT* expression was progressively decreased as HEY cells became more confluent. Therefore, to address whether the dbcAMP-associated increase in *c-KIT* mRNA expression was simply a consequence of HEY cell confluency, in a preliminary experiment HEY cells were grown to approximately 70% confluence (at which point *c-KIT* expression should be very low) and then treated with dbcAMP for 24 h. Northern blot analysis indicated that the dbcAMP treatment still increased *c-KIT* mRNA in HEY cells at high cell confluency (data not shown), suggesting that this effect of cAMP is independent of cell confluency. HEY cell SCF expression was upregulated in the highest confluency cultures, an explanation for which is not obvious since there would be no functional relevance to increased SCF levels in the absence of *c-KIT* expression.

The changes in HEY cell *c-KIT* expression with cell confluency were not observed with OVCA 429 cells, suggesting that the results in HEY cells cannot be extended to other ovarian cancer cells at this point. Differences in the *in vitro* responses of these two ovarian carcinoma cell lines may be due, in part, to their different origin: the HEY cell line was derived from a murine xenograft of a human cystadenocarcinoma of the ovary (Buick *et al.*, 1985), while OVCA 429 cells were established from the peritoneal ascites of an adenocarcinoma (Berchuck *et al.*, 1992).

The observations that dbcAMP-mediated inhibition of HEY cell proliferation was temporally associated with an increase in *c-KIT* and, conversely, that TGF- α -mediated

stimulation of proliferation correlated with decreased *c-KIT*, suggests that KIT might suppress proliferation of these cells. This is in direct contrast to the well documented role of KIT:SCF in stimulating proliferation and survival of both normal and cancer cells (detailed in Chapter 1). In an effort to determine whether the KIT:SCF interaction in HEY and OVCA 429 cells could modulate their proliferation, the cells were cultured in the presence of the SR-1 neutralizing antibody and/or exogenous SCF. Proliferation of both of these ovarian carcinoma cell lines was not affected by blocking activation of the KIT receptor nor by SCF stimulation in serum-free media. The dbcAMP-mediated inhibition of HEY cell proliferation was also not prevented by concurrent exposure to SR-1 (Keszthelyi *et al.*, submitted). The inability to modulate proliferation can be explained by the failure of these cells to localize KIT protein to the plasma membrane, since both SR-1 and SCF are targeted to cell surface KIT receptors. In contrast to the HEY cells, the proliferation of a control cell line, MO7/e, which is dependent on SCF activation of the KIT receptor for its growth, was significantly decreased in the presence of SR-1 (Keszthelyi *et al.*, submitted).

Despite the fact that the *c-KIT* expressed by both primary ovarian tumor cells *in vivo* (Chapter 3) and ovarian carcinoma cells (this study) was predominantly the membrane-associated isoform, the cytoplasmic localization of KIT protein with a predominant perinuclear staining suggests that KIT receptor is not being properly trafficked to the membrane. A possible explanation for this would be the presence of one or more mutations in the *c-KIT* expressed by ovarian cancer cells that might affect the ability of KIT protein to localize to the cell membrane. Mutations of the *c-KIT* gene have been reported in other cancers, including leukemias of the mast cell lineage (Furitsu *et al.*, 1993; Nagata *et al.*, 1995;

Longley *et al.*, 1996; Ashman *et al.*, 2000) and gastrointestinal stromal tumors (Hirota *et al.*, 1998; Nakahara *et al.*, 1998). Indeed, sequencing of the *c-KIT* produced by one of the ovarian tumors reported in Chapter 3 revealed three point mutations, one of which was in the transmembrane domain of the protein. Determination of the sequence of *c-KIT* expressed by HEY cells is currently under investigation. However, since HEY cells and primary ovarian tumor cells co-express KIT and SCF, the possibility exists that an intracrine KIT:SCF interaction may regulate ovarian cancer cell proliferation, and this is addressed in Chapter 5.

Chapter 5 - A Function of KIT in Ovarian Cancer Cells

5.1 Introduction

The previous two chapters provided a few lines of evidence that the KIT:SCF interaction in human ovarian carcinoma cells could be negatively regulating the proliferation of those cells. First, patients with malignant epithelial ovarian cancers whose tumors expressed c-*KIT* were found to have a longer disease-free survival compared to patients whose tumors did not express c-*KIT*. This result suggests that activated KIT in those patients' tumor cells could be decreasing the proliferation and/or increasing the chemosensitivity of those cells. Second, treatment of HEY ovarian carcinoma cells with TGF- α resulted in increased proliferation of those cells concomitant with a dramatic decrease in c-*KIT* expression. TGF- α -mediated growth stimulation of ovarian carcinoma cells has been associated with upregulation of both its own mRNA (Jindal *et al.*, 1995) and c-*myc* gene expression (Park, 1997), so it would make sense for TGF- α to downregulate c-*KIT* if activated KIT inhibits growth. Lastly, the dbcAMP-mediated growth suppression of HEY cells was temporally associated with a significant increase in c-*KIT* expression, again suggesting that activated KIT can inhibit ovarian cancer cell growth.

Although activated KIT has been demonstrated to increase the proliferation of several human cancer cell types, such as leukemia (Crosier *et al.*, 1993; Sekido *et al.*, 1993), lung (Sekido *et al.*, 1993; Papadimitriou *et al.*, 1995; Krystal *et al.*, 1996) and prostate (Savarese *et al.*, 1998), there is also evidence that the KIT:SCF interaction can negatively regulate the proliferation of breast cancer cells (Nishida *et al.*, 1996) and melanoma cells (Huang *et al.*,

1996).

Initial attempts to determine whether the KIT:SCF interaction in ovarian carcinoma cells influenced their proliferative rate (Chapter 4) were unsuccessful, presumably due to an exclusively cytoplasmic expression of KIT protein. As has been previously discussed (Chapter 3), cytoplasmic subcellular localization of KIT protein has been reported for many cancer cell types. Co-expression of KIT and SCF in ovarian carcinoma cells and primary ovarian tumor cells raises the intriguing possibility that interaction of SCF with KIT may occur intracellularly in these cells. Thus, the specific objectives of Chapter 5 were: (1) to determine if cytoplasmic KIT is active in HEY ovarian carcinoma cells; (2) to attempt to knock out HEY KIT expression using an antisense oligonucleotide approach and assess proliferation; (3) to transfect *c-KIT* into KIT-negative ovarian carcinoma cells and assess proliferation; and (4) to increase the endogenous level of KIT in HEY cells by transfecting *c-kit* into these cells and assess proliferation.

5.2 Materials and Methods

5.2.1 Determination of KIT tyrosine phosphorylation and kinase activity in HEY cells

HEY cells were seeded at 5×10^5 cells per 100 mm dish in 5 ml of α -MEM + 10% FCS, either in the absence or presence of 2 mM dbcAMP, for a total of 24 h. During the last hour of culture, the cells were incubated with 2 mM sodium orthovanadate, a general phosphatase inhibitor, in order that the phosphorylation status of HEY cell proteins would

be maintained. Total protein lysates were subsequently collected from the cells, quantified, and western blot analysis using 50 µg of each lysate was carried out. Immunodetection of tyrosine-phosphorylated proteins was done first by probing the membrane with a mouse anti-human phosphotyrosine (PY) MAbs (Transduction Laboratories, Lexington, KY; 1 µg/ml). The positive control for tyrosine-phosphorylated proteins was a total protein lysate from EGF-R-positive A431 human epidermoid carcinoma cells (Transduction Laboratories) that had been stimulated with 100 ng/ml EGF for 5 min., a treatment that dramatically increases the tyrosine phosphorylation of EGF-R and certain other cellular proteins. Following visualization of tyrosine-phosphorylated proteins, the membrane was stripped using a solution containing 62.5 mM Tris-HCl pH 6.7, 2% SDS, and 100 mM 2-mercaptoethanol, for 30 min with agitation at 50°C. The membrane was then re-probed for KIT protein expression as previously described.

To assess whether HEY cells possessed active KIT kinase, an *in vitro* autophosphorylation assay was performed. KIT protein was first immunoprecipitated, as described in section 2.5, from 1 ml of total protein lysate that had been extracted from 8×10^6 HEY cells. Following the immunoprecipitation, the beads were washed twice with Frack's buffer and then twice with kinase buffer [20 mM 2(N-morpholino)ethanesulfonic acid pH 6.5, 10 mM MgCl₂, 2 mM MnCl₂]. The beads were next incubated in 50 µl of kinase buffer containing 50 µCi of γ -³²P-ATP for 30 min at RT. The beads were washed twice with 1 ml of Frack's buffer to remove unincorporated radiolabel, resuspended in 50 µl of 2X Sample Buffer (0.125 M Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 5% 2-mercaptoethanol, 0.05% bromophenol blue), boiled 3 min, and then run in a 5% polyacrylamide gel. The gel was

subsequently fixed in a 45:10:45 mixture of methanol:acetic acid:ddH₂O for 10 min at RT, rinsed in ddH₂O, and exposed and imaged as described for northern blots in section 2.3.

5.2.2 Plasmid vectors

A number of plasmid vectors were used in single and co-transfection studies described in the subsequent sections. Two different *c-KIT*-containing vectors were used: pKJ136 (Figure 23A; generously provided by Dr. M. McBurney, Ottawa, ON), has a phosphoglycerate kinase (pgk) promoter driving expression of a 5.1 kb human *c-KIT* cDNA, and a hygromycin resistance gene; and pECE*kit* (Figure 23B; kindly provided by Dr. J. Bell, Ottawa, ON), which has expression of a 3.7 kb murine *c-kit* cDNA driven by an SV40 promoter. The pKJ136 *c-KIT* cDNA lacked the 12 bp encoding the 4 amino acid KIT proteolytic cleavage site, while the *c-kit* mRNA expressed from pECE*kit* contained those 12 bp. Since the pECE*kit* vector contains only an ampicillin resistance gene which is not suitable for use as a selectable marker in mammalian cells, it was co-transfected with a vector containing a hygromycin resistance gene (Figure 24A; also provided by Dr. M. McBurney).

In addition to the *c-kit* vectors, a few other plasmid vectors were used as controls in these studies. The backbone vectors were pKJ90 and pECE, which are identical to pKJ136 and pECE*kit*, respectively, except for the absence of the *c-kit* cDNA. Additionally, parallel transient transfection of pDM2, a vector which has the pgk promoter driving expression of the *lacZ* gene (Figure 24B), was always done in order to get an estimate of the transfection efficiency. *LacZ* encodes the β -galactosidase (β -gal) enzyme and cells which express β -gal

Figure 23

Schematic representation of the two c-*KIT* expression vectors used in these studies. The pKJ136 vector (A) contained a full-length, 5.1 kb human c-*KIT* cDNA cloned into the *Sall* and *NotI* sites within the multiple cloning site. Expression of c-*KIT* was driven by a phosphoglycerate kinase (pgk) promoter. The presence of an internal ribosomal entry site (IRES) allowed for the translation of a hygromycin resistance (HygTK) mRNA from the same vector. A second vector, pECE*kit* (B), contained a 3.7 kb murine c-*kit* cDNA cloned into the *EcoRI* and *XbaI* sites of the pECE multiple cloning site. Expression of the murine c-*kit* was driven by an SV40 promoter. The pKJ136 c-*KIT* cDNA lacked the 12 bp encoding the 4 amino acid KIT proteolytic cleavage site, while the c-*kit* mRNA expressed from pECE*kit* contained those 12 bp.

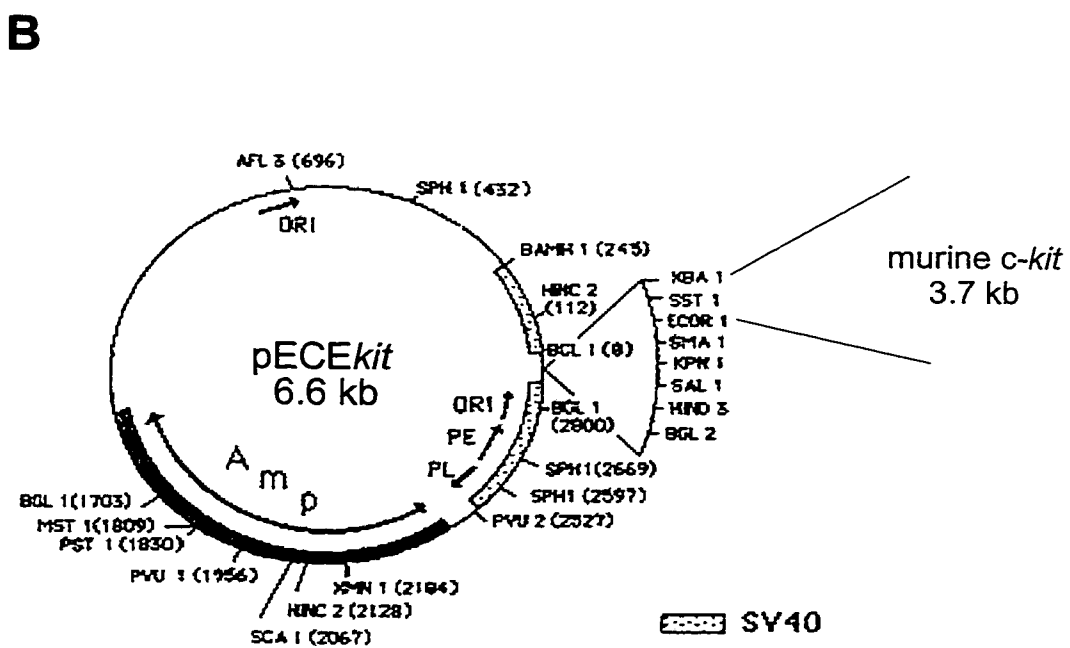
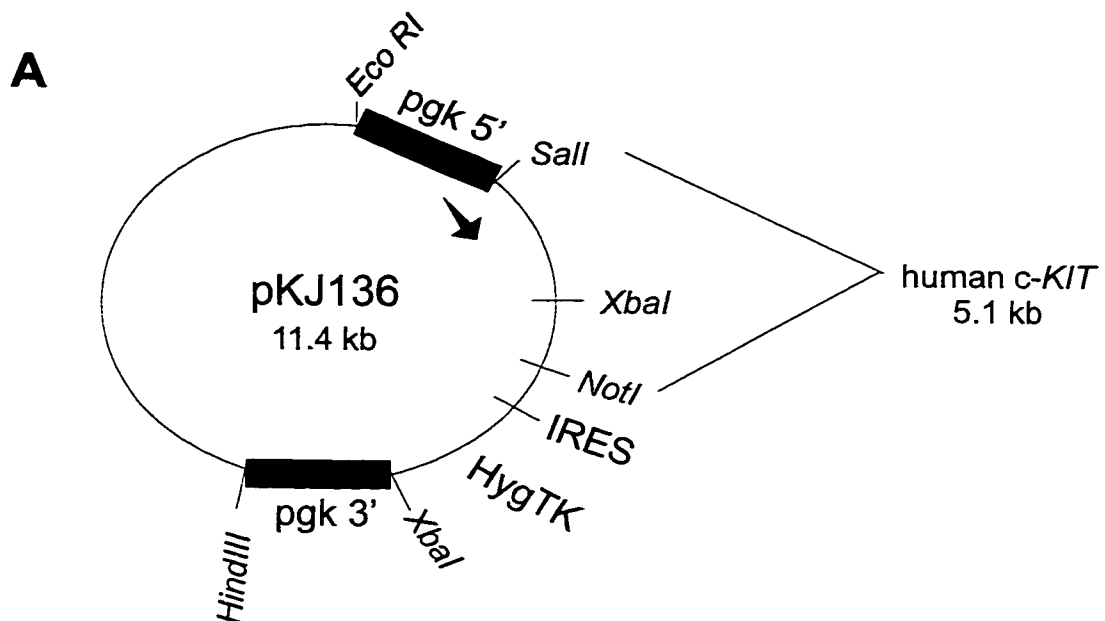
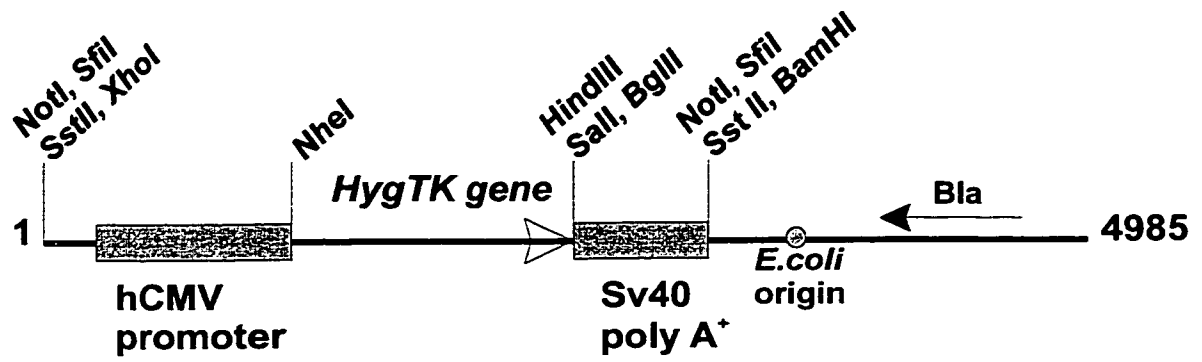


Figure 24

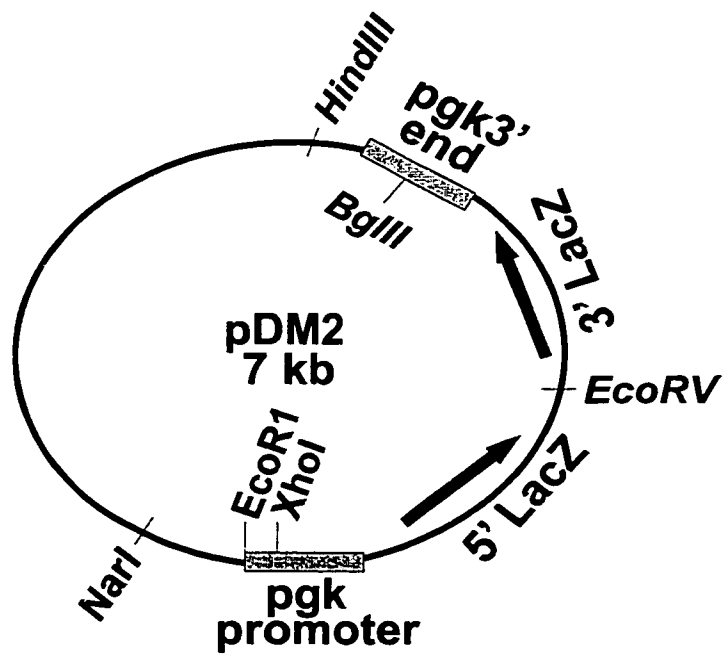
Schematic representation of two additional plasmid vectors used in these studies. (A)

A linear representation of a vector containing a hygromycin resistance gene with expression driven by a human cytomegalavirus (hCMV) promoter. This vector was used in co-transfection studies of the pECE*kit* vector, which lacked a eukaryotic antibiotic resistance gene, to allow for the *in vitro* selection of stably transfected cells. Determination of the transfection efficiency in c-*KIT* transfection studies was done by parallel transfection of the cells using the pDM2 vector (B) containing the *lacZ* gene whose expression was driven by the phosphoglycerate kinase (pgk) promoter. *LacZ* encodes β -galactosidase, an enzyme which can metabolize an exogenously provided substrate (X-gal) to yield a blue reaction product in *lacZ*-transfected cells.

A



B



can metabolize an exogenously-provided substrate, 5-bromo-4-chloro-3-indolyl-B-D-galactopyranoside (X-gal; BioShop Canada Inc., Burlington, ON), to yield a blue reaction product. X-gal staining of cells following their transfection with pDM2 was accomplished by aspirating the culture medium, rinsing with cold PBS, fixing for 30 min at 4°C with 0.2% glutaraldehyde (in 100 mM sodium phosphate buffer, pH 7.4), rinsing twice with PBS, and then adding X-gal stain (8 mM potassium ferrocyanide, 8 mM potassium ferricyanide, 1 mM MgSO₄, 1 mg/ml X-gal in 100 mM sodium phosphate buffer, pH 7.4). The cells were stained in the X-gal solution overnight at 37°C and the percentage of β -gal-expressing blue cells was estimated 24 h later. For all of the experiments reported herein, the estimated transfection efficiency was ≤ 20 -30%.

5.2.3 Stable transfection of c-*KIT* into human ovarian carcinoma cells

Experiments to obtain human ovarian carcinoma cells that stably expressed c-*KIT* were carried out using A2780-s and A2780-cp cells, which do not express c-*KIT* but do express SCF (Figure 13). A2780-s cells are sensitive to cisplatin, while A2780-cp cells are a cisplatin-resistant cell line. A2780-s and -cp cells were seeded at 0.35 - 0.75×10^6 cells per 60 mm dish 24 h prior to transfection. The cells were transfected with 1.3 pmol of pDM2, pKJ90, or pKJ136 using 16 μ l of Lipofectamine Reagent (Gibco) according to the manufacturer's instructions (this DNA:Lipofectamine ratio had been previously determined to provide the maximum transfection efficiency in these cells using the pDM2 vector). Briefly, for each transfection 1.3 pmol of vector DNA and 16 μ l of Lipofectamine were separately diluted into 300 μ l of serum-free medium. The two solutions were combined,

mixed gently, and incubated at room temperature for 45 min to allow the DNA-liposome complexes to form. The DNA-liposome solution was then further diluted to 2.5 ml with serum-free media, and added to cells which were 50-80% confluent and which had been rinsed with serum-free media. After 5 h of transfection in serum-free media, 2.5 ml of medium which contained twice the normal amount of serum was added to each dish and the transfections were continued for a total of 24-48 h. The pDM2-transfected cells were subsequently X-gal-stained to obtain an estimate of the transfection efficiency while, for selection of stable transfectants, the pKJ90- and pKJ136-transfected cells were trypsinized and replated in 100 mm dishes under the selection pressure of 100 µg/ml hygromycin (Hygromycin B; Boehringer Mannheim; 50 mg/ml stock in sterile PBS, stored at 4°C). The cells were selected in hygromycin for approximately 3 weeks, at which time individual colonies of cells were picked and expanded in culture.

Lipofectamine-mediated co-transfection of the pECE + hygTK and pECE*kit* + hygTK vectors into A2780-s and -cp for the purpose of obtaining stable *c-kit* transfectants was done as described for the single vector transfections above, keeping the total amount of DNA constant, i.e., 0.65 pmol pECE*kit* + 0.65 pmol hygTK was used in conjunction with 16 µl of Lipofectamine.

Determination of *c-KIT* mRNA and/or KIT protein in hygromycin-resistant clones was done by collecting the RNA from the cells and performing northern blot analysis (see section 2.3), or by collecting total protein lysates and performing western blot analysis (see section 2.5), or by carrying out immunocytochemical analysis of transfectants grown on coverslips (see section 2.6.2).

The cloning efficiency of A2780-cp cells transfected with pECE + hygTK versus pECE*kit* + hygTK was determined by plating (4x100 mm dishes per vector type) an equal number of transfected cells and, approximately 2 weeks later, simply counting the number of hygromycin-resistant colonies comprised of ≥ 50 cells that grew in each plate. The proliferation of stably transfected pooled populations of A2780-cp cells and individual clones was assessed by seeding parental untransfected and/or pECE-transfected and pECE*kit*-transfected cells at either 1×10^5 cells in triplicate 35 mm dishes or 3×10^5 cells in triplicate 60 mm dishes (with continued hygromycin selection pressure) for 48 h. Viable and dead cells were then counted using trypan blue exclusion and hemacytometry as described in section 2.2.1.

5.2.4 Transient transfection of *c-kit*

A2780-s cells were transiently transfected in order to assess the subcellular localization of Kit protein expressed by the transfectants. The cells were seeded 24 h prior to transfection at 0.5×10^6 cells per 60 mm dish (each dish contained two gelatin-coated coverslips). Lipofectamine-mediated transfection of 1.3 pmol of pDM2, pKJ90, pKJ136, or pECE*kit* was carried out as described for the stable transfections. After 24 h, coverslipped cells were processed for immunocytochemical detection of KIT protein as described in section 2.6.2.

HEY cells and NIH 3T3 mouse fibroblast cells were also transiently transfected with the pECE and pECE*kit* vectors and the proliferation of parental untransfected, pECE- or pECE*kit*-transfected cells was determined. The cells were seeded at 1×10^5 cells in triplicate

60 mm dishes 24 h prior to transfection. Transfection of the cells was done using 3 µg DNA and 12 µl Lipofectamine (HEY) or 4 µg DNA and 16 µl Lipofectamine (NIH 3T3) for 24 h, then the transfected cells were trypsinized, pooled, and replated at 5×10^4 cells in triplicate 60 mm dishes. After 48 h of culture, proliferation of the cells was determined using hemacytometry as detailed in section 2.2.1.

5.2.5 Culture of HEY cells with oligonucleotides

HEY cells were first tested for their ability to take up an irrelevant, fluorescent oligonucleotide (oligo). The cells were seeded at 2×10^4 cells in 350 µl α -MEM + 10% FCS, in duplicate wells of a 24-well plate, and each well contained a sterile 0.15% gelatin-coated 12 mm coverslip (Fisher). After 24 h, the cells were exposed to 0.5-2.5 µg of a FITC-conjugated 25-mer oligo (generously provided by Dr. H. Clarke, Montreal, PQ) in the absence or presence of 2-6 µl Fugene™ 6 Transfection Reagent (Boehringer Mannheim) according to the manufacturer's instructions (control wells received no oligo and/or Fugene). Briefly, the Fugene was diluted directly into serum-free medium to a final volume of 100 µl and incubated for 5 min at room temperature. The diluted Fugene was added, dropwise, to a second tube containing the fluorescent oligo, the tube was gently tapped to mix the components, and then incubated for 15 min at room temperature. The oligo:Fugene mixture was then added, dropwise, to the cultured HEY cells (the 100 µl was divided between duplicate wells to yield a final volume of 400 µl per well). The final oligo (µg):Fugene (µl) ratios were, therefore, 0.5:2, 1:3, and 2.5:6. The cells were incubated for an additional 24 h in the presence of the oligo:Fugene complexes. To assess the uptake of the FITC-

conjugated oligo by HEY cells, the coverslipped cells were simply rinsed in S-PBS, fixed in Lana's fix, and mounted in 3 μ l of anti-fade mounting medium. Digital images were obtained as described in section 2.6.1.

In an effort to decrease or abolish HEY cell KIT expression, the cells were cultured as described above with 1.25, 2.5, 5, or 10 μ M of an antisense *c-KIT* oligo or 10 μ M of the corresponding sense oligo in the absence or presence of 6-48 μ l of Fugene [oligo (μ g):Fugene (μ l) were 2.6:6, 5.1:12, 10.4:24, or 20.8:48]. The 18-mer *c-KIT* oligo sequences were the same as those reported by Ratajczak *et al.* (1992): antisense 5'-GCC GCG AGC GCC TCT CAT-3' and sense 5'-ATG AGA GGC GCT CGC GGC-3' (Gibco). The oligos were phosphorothioate-modified for stability, and both were reconstituted in sterile ddH₂O, to final concentrations of 1.317 μ g/ μ l (antisense) and 1.144 μ g/ μ l (sense). The oligo sequences were run through BLASTN GenBank and neither sequence contained homology to any mRNA other than *c-KIT*. After 18 h in the presence of oligos, the coverslipped cells were assessed for KIT protein expression by immunocytochemistry as described in section 2.6.2.

The *c-KIT* oligo experiments were also carried out with Lipofectamine, as described in section 5.2.2, using the following ratios of oligo (μ g):Lipofectamine (μ l): 2.6:8; 5.1:8; 10.4:16; and 20.8:16. Following 6 and 18 h of incubation, the coverslipped cells were processed for immunocytochemical detection of KIT protein.

5.2.6 Statistical Analyses

The number of pECE-transfected versus pECE*kit*-transfected A2780-cp colonies was statistically compared using a Student's T-test with significance inferred at $p \leq 0.05$. Statistical analysis of the proliferation of parental untransfected, backbone vector-transfected, and c-*kit*-transfected A2780-cp, HEY, and NIH 3T3 cells was performed as described in section 2.7.

5.3 Results

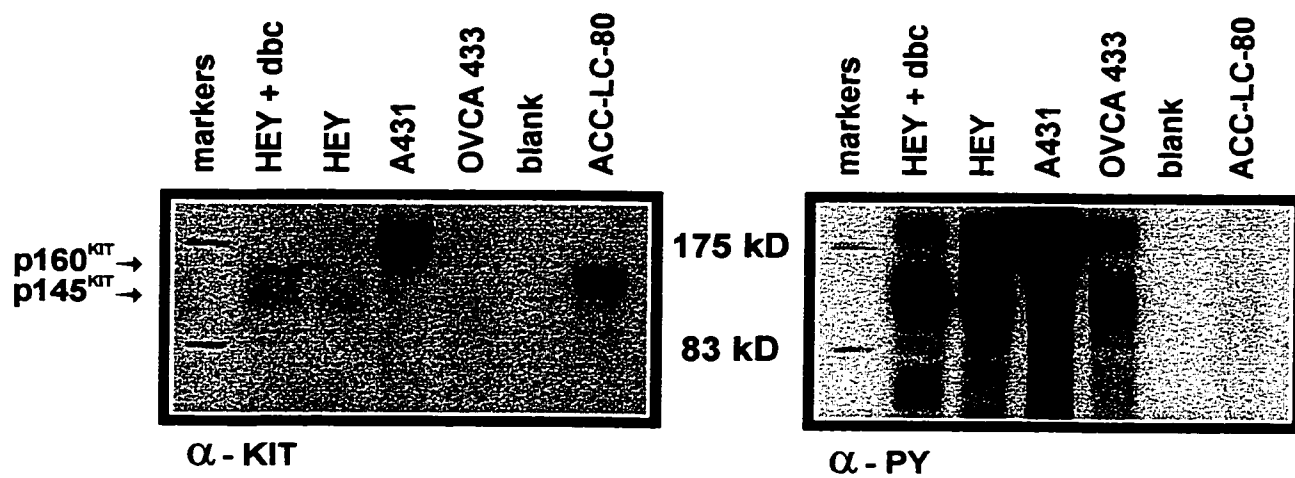
5.3.1 Cytoplasmic KIT protein is active

Since KIT protein was detected in the cytoplasm and not at the plasma membrane of primary ovarian tumor cells (Chapter 3) and ovarian carcinoma cells (Chapter 4), it was pertinent to determine whether cytoplasmic KIT could be functional in these cells. Therefore, the tyrosine phosphorylation status of KIT and its intrinsic kinase activity were assessed in HEY cells. Analysis of total protein lysates from cultured HEY cells revealed that the cells contained tyrosine-phosphorylated proteins corresponding in size to the 145 kD and 160 kD KIT proteins (Figure 25A). To determine if the KIT protein in HEY cells possessed any kinase activity, an *in vitro* autophosphorylation assay was done by incubating immunoprecipitated KIT protein in the presence of radiolabeled ATP. Figure 25B shows that the p145^{KIT} protein in HEY cells demonstrated kinase activity and became radiolabeled under those conditions.

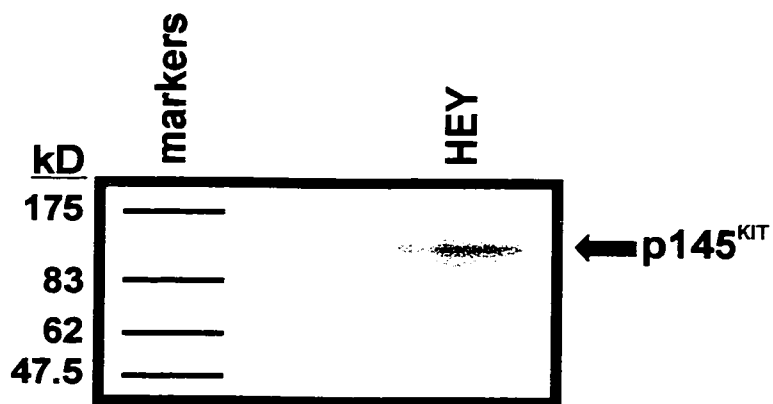
Figure 25

KIT protein in HEY cells is tyrosine-phosphorylated and kinase-active. (A) HEY cells were cultured in the absence (HEY) or presence of 2 mM dibutyryl cyclic AMP (HEY + dbc) for 24 h. During the last hour of culture, the cells were treated with 2 mM sodium orthovanadate, a phosphatase inhibitor, so that the phosphorylation status of the proteins would be maintained. Western blot analysis to determine if KIT protein was tyrosine-phosphorylated was accomplished using 50 µg of protein lysates and consecutive incubation of the membrane with an anti-phosphotyrosine MAb (α -PY) followed by an anti-KIT MAb (α -KIT). An A431 cellular protein lysate was used as the positive control for tyrosine-phosphorylated proteins. Proteins corresponding to the p145 and p160 KIT proteins were tyrosine-phosphorylated in HEY cells, and dbc treatment of those cells increased the amount of KIT tyrosine-phosphorylated proteins. The abundant high molecular weight protein that is tyrosine-phosphorylated in the control A431 lysate is the EGF-R. To determine if HEY cell KIT was an active kinase (B), KIT protein was immunoprecipitated from 8×10^6 HEY cells and subjected to an *in vitro* autophosphorylation assay as described in the Materials and Methods. HEY cell KIT protein was an active kinase as demonstrated by its ability to autophosphorylate p145^{KIT} *in vitro*.

A



B



5.3.2 Disrupting the KIT:SCF interaction in HEY cells

In an effort to determine whether the KIT:SCF interaction in HEY cells modulated their proliferation, an antisense oligo approach was employed to disrupt the expression of KIT in these cells. First, a “test” FITC-conjugated oligo was used to determine whether HEY cells were capable of taking up an oligo. The test oligo was a 25-mer, FITC-conjugated oligo which had been previously shown to be taken up by murine ovarian granulosa cells cultured in the presence of Fugene (Dr. H. Clarke, personal communication). Although the HEY cells did appear to take up the FITC-conjugated test oligo, as evidenced by a more intense green fluorescence compared to control, untreated cells (Figure 26*d*), this was not a conclusive result since HEY cells demonstrated autofluorescence under the FITC filter. Nonetheless, experiments using *c-KIT* antisense and sense oligos were carried out. However, no decrease in KIT protein expression was observed in HEY cells cultured with the antisense *c-KIT* oligo (compared to HEY cells cultured without oligo or with the sense *c-KIT* oligo; data not shown), despite using Fugene and Lipofectamine reagents to enhance the cellular uptake of the oligos.

5.3.3 Transfection of *c-KIT* into A2780 ovarian carcinoma cells

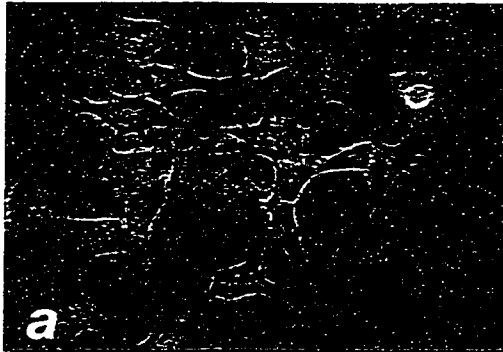
Experiments in which *c-KIT* was transfected into ovarian carcinoma cells that did not express endogenous *c-KIT* were carried out to ascertain whether enforced expression of *c-KIT* would affect the proliferation of the cells. Two ovarian carcinoma cell lines, A2780-s and A2780-cp, were transfected with a human *c-KIT* construct (pKJ136; Figure 23A) and attempts were made to obtain stably transfected cells that expressed *c-KIT*. Although

Figure 26

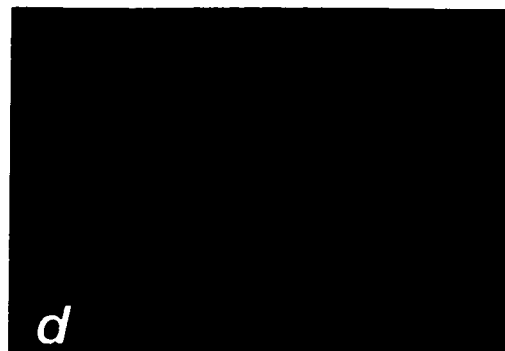
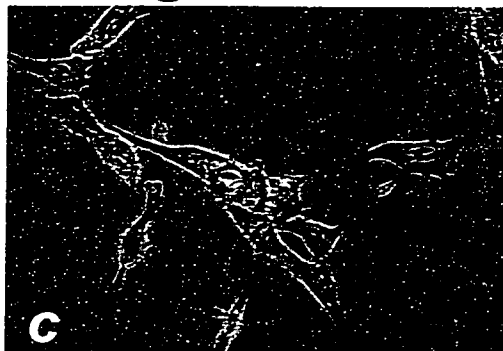
HEY ovarian carcinoma cells show uptake up a FITC-conjugated oligonucleotide.

HEY cells were grown in the absence (control) or presence of 2.5 μ g of an FITC-conjugated, 25-mer oligonucleotide (+ oligo) and Fugene transfection reagent for the second 24 h of a 48 h culture period. Panels *a* and *c* are the phase contrast views of the fluorescent images in panels *b* and *d*. HEY cells appeared to take up the FITC-conjugated oligo as evidenced by green fluorescence in the cytoplasm of cells. Magnification 200X.

control



+ oligo



hygromycin-resistant clones were obtained with both cell lines, none of these clones expressed *c-KIT* as assessed by northern and western blot analyses (data not shown).

To determine if *c-KIT* was at least being transiently expressed in the transfected cells, A2780-s cells were transiently transfected with the human *c-KIT* construct and 24 h later were stained for KIT protein expression. The KIT protein expressed by the A2780-s cells transfected with pKJ136 was neither membrane-associated nor cytoplasmic, but rather was predominantly expressed as a bright, peri-nuclear “glob” within the cells (Figure 27*d*, arrowheads). An inability to obtain stable *c-KIT*-expressing clones using the pKJ136 vector may have been caused by a toxicity associated with the manner in which KIT protein was expressed in the cells. In contrast to the localization of human KIT protein in the A2780-s cells, cells expressing the murine *c-kit* from the pECE*kit* vector showed clear membrane-associated (Figure 27*e*, arrowheads) and cytoplasmic Kit protein. Therefore, the pECE*kit* vector was used in all subsequent transfection experiments.

Microscopic observation of the growth of transfectants expressing the pECE backbone vector versus the pECE*kit* vector revealed some interesting behavior *in vitro*. Backbone vector-transfected A2780-cp cells grew more rapidly than did those cells transfected with pECE*kit*. The *c-kit*-transfected cells formed fewer colonies than did the backbone vector-transfected cells and, over time in culture, many of the *c-kit* transfectant colonies died. Actual quantification of the number of colonies formed by A2780-cp cells transfected with the pECE-based vectors revealed a statistically significant 7-fold decrease in the number of colonies (≥ 50 cells) formed by *c-KIT*-transfected cells compared to backbone vector-transfected cells (102 ± 36 colonies versus 764 ± 216 colonies, respectively;

Figure 27

A2780-s ovarian carcinoma cells show aberrant localization of KIT protein when transfected with human c-*KIT* but not murine c-*kit*. A2780-s cells were transfected with either the pKJ136 vector which expresses human c-*KIT* (+ hc-*KIT*) or the pECE*kit* vector which expresses murine c-*kit* (+ mc-*kit*), in the presence of Lipofectamine transfection reagent for 24 h. Immunocytochemical detection of KIT protein was done using an anti-human KIT polyclonal antibody (*a-d*) or an anti-mouse Kit MAb (*e*). ACC-LC-80 lung cancer cells were used as the positive control for KIT protein expression (*a*), and untransfected (untrans; *b*) or pKJ90 backbone vector-transfected (-hc-*KIT*; *c*) A2780-s cells were used as the negative controls for Kit protein expression. Panel *f* shows the amount of background staining in pKJ136-transfected cells that was due to the fluorochrome-conjugated secondary antibody alone. A2780-s cells transfected with pKJ136 expressed human KIT protein as a bright, peri-nuclear glob (arrowheads, *d*), while cells expressing murine Kit vector showed membrane-associated (arrowheads, *e*) and cytoplasmic Kit protein expression similar to that observed for the control ACC-LC-80 cells. Magnification 200X.

ACC-LC-80



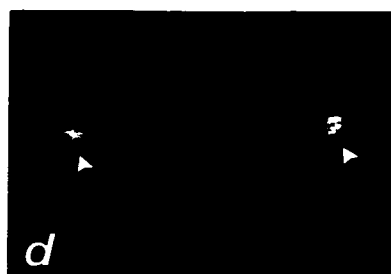
untrans



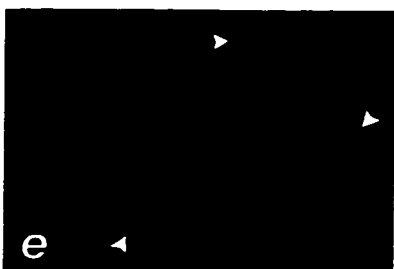
**pKJ90
(- hc-*KIT*)**



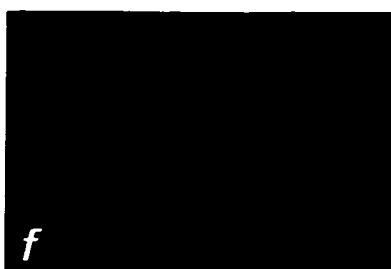
**pKJ136
(+ hc-*KIT*)**



**pECE*kit*
(+ mc-*kit*)**



no 1° Ab



$p < 0.05$). In fact, initial attempts to obtain individual, stable pECE*kit*-transfected A2780 clones that expressed Kit were unsuccessful.

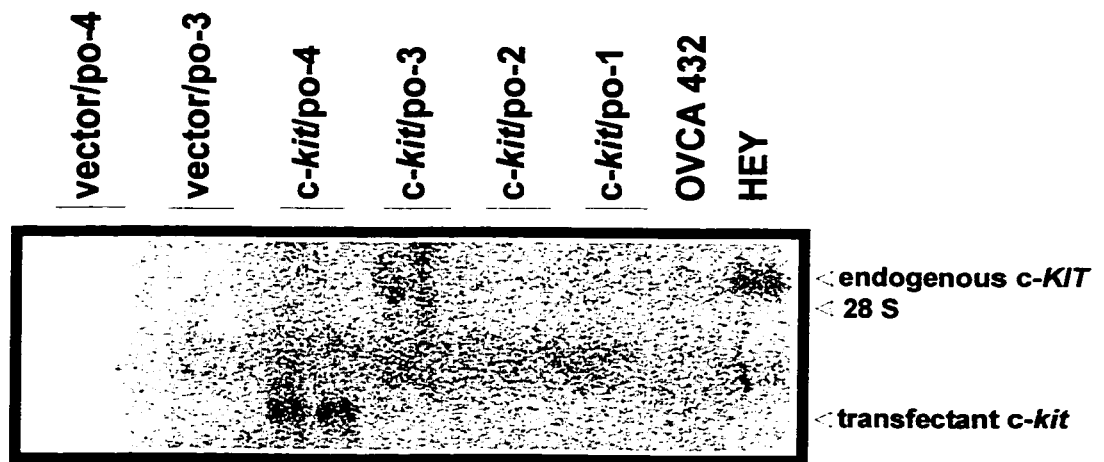
However, northern blot analysis of the *c-kit* mRNA expression by *pooled* colonies of pECE*kit*-transfected A2780-cp cells revealed that 1 of 4 pooled *c-kit* transfectants (*c-kit*/po-4) expressed *c-kit* mRNA of the expected 3.7 kb size (Figure 28A). When this population was examined for Kit protein expression by immunocytochemistry, it was estimated that 20-30% of the cells showed membrane-associated and/or cytoplasmic Kit expression (Figure 28B, panels *b* and *c*). Most interestingly, the *c-kit*/po-4 population of cells demonstrated an approximate 40% decrease in proliferation ($p < 0.05$) compared to either the parental untransfected A2780-cp cells or the backbone vector-transfected cells (Figure 29).

Subsequently, individual stably-transfected *c-kit* clones were derived from the *c-kit*/po-4 population by replating the cells under hygromycin selection pressure. Selected clones were screened for Kit expression by immunocytochemistry, and 13 of the 50 clones screened (26%) were found to express Kit (localization of Kit was the same as for the *c-kit*/po-4 pooled population shown in Figure 28B). However, only 20-30% of the total cell population of each of the 13 stable clones expressed Kit. Eleven of the 13 stable *c-kit* clones were also examined for *c-kit* mRNA expression, and all expressed the 3.7 kb transfectant *c-kit* mRNA (Figure 30A). The first experiment to determine if, like the *c-kit*/po-4 pooled population, an individual Kit-expressing stable clone would proliferate more slowly in culture revealed that stable clone Kit6-1 did indeed show an approximate 40% reduction in cell number compared to a single clone that was stably transfected with just the backbone vector (Figure 30B). However, when the same proliferation experiment was repeated with

Figure 28

Expression of membrane-associated and cytosolic Kit protein in *c-kit*-transfected ovarian carcinoma cells. A2780-cp cells were co-transfected with a vector containing the murine *c-kit* gene and a vector containing the hygromycin resistance gene. Cells were selected in hygromycin for approximately two weeks, then the expression of *c-kit* mRNA in pooled populations of *c-kit*-transfected cells was done by northern blot analysis (A) using 15 µg of total cellular RNA and a [³²P]-labeled human *c-KIT* cDNA. HEY cells were used as the positive control for *c-KIT* mRNA expression, while OVCA 432 cells served as the negative control. One of four pooled populations of *c-kit*-transfected A2780-cp cells, *c-kit*/po-4, showed expression of the 3.7 kb transfectant *c-kit* mRNA. A2780-cp cells transfected with just the backbone vector showed no expression of *c-kit* mRNA (vector/po-3 and -4). Immunocytochemical localization of Kit protein in the *c-kit*/po-4 pooled population (B) was accomplished using a rat anti-mouse MAb, ACK2, and a FITC-conjugated secondary antibody. Parental, untransfected A2780-cp cells (not shown), and backbone vector only-transfected cells (*a*) showed no detectable Kit protein staining, while the *c-kit*-transfected pooled population contained cells expressing membrane-associated (arrow) and/or cytosolic Kit protein (*b*, *c*). Magnification 200X.

A



B

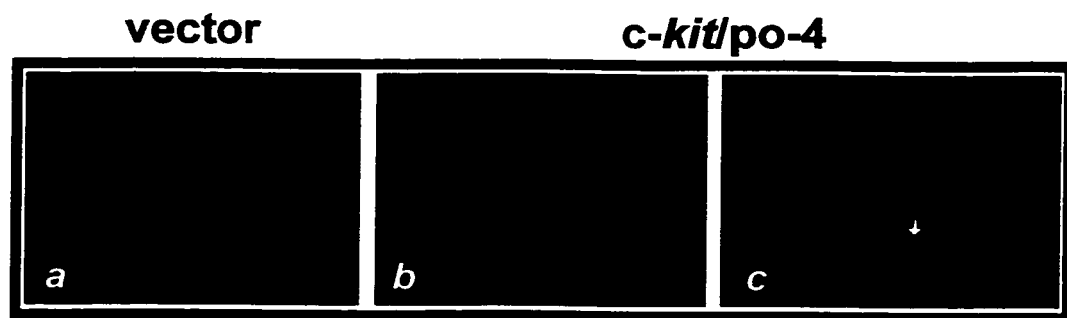
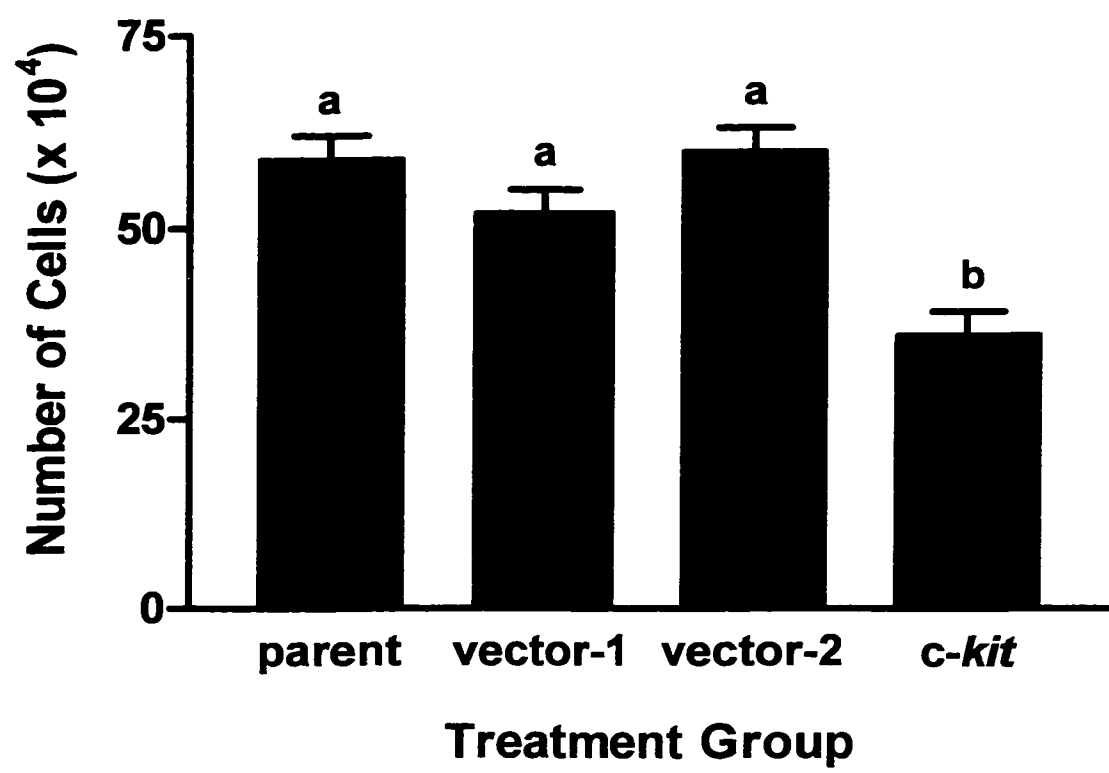


Figure 29

Enforced expression of Kit protein in ovarian carcinoma cells causes decreased proliferation. Parental untransfected A2780-cp cells (parent), backbone vector-transfected cells (two different pooled populations, vector-1 and vector-2) and *c-kit*-transfected cells (*c-kit*) were cultured for 48 h in their normal 15% serum-containing medium. Cellular proliferation was assessed using hemacytometry. Values are the mean $\pm$ SEM from 3-4 experiments done in triplicate. Different lowercase letters above the bars indicate significant differences between treatment groups. Kit-expressing A2780-cp cells demonstrated a significantly decreased rate of proliferation compared to parental untransfected cells and vector only-transfected cells ($p<0.05$).



stable clone Kit6-1 and two other stable clones *after the clones had been frozen and thawed*, none of the three clones demonstrated any decrease in proliferation or survival relative to parental untransfected A2780-cp cells or a backbone vector-transfected stable clone (Figure 30C).

5.3.4 Transient expression of Kit protein in HEY cells

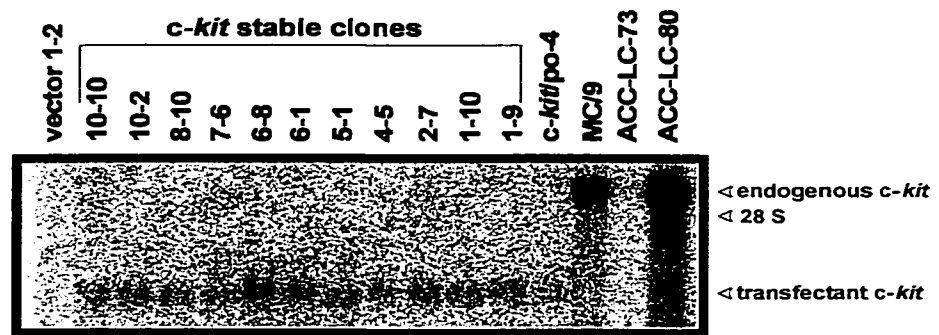
As the attempts to knock out *c-KIT* expression in HEY cells using an antisense oligo approach had been unsuccessful, and since HEY cells express relatively low amounts of *c-KIT* (compared to, for example, MC/9 mast cells), we next determined if expressing more Kit protein in HEY cells would affect their proliferation. As shown in Figure 31A, transient transfection of murine *c-kit* into HEY cells resulted in a significant decrease in the proliferation of those cells compared to untransfected control HEY cells ($p<0.0001$) or backbone vector-transfected cells ($p<0.001$). *C-kit* expression did not appear to be affecting the survival of the HEY cells, however, since there was no difference in the number of floating cells in transfected versus control cell cultures.

To ensure that the decreased proliferation of HEY and A2780-cp cells transfected with *c-kit* was not simply a non-specific toxic effect of the *c-kit* vector, NIH 3T3 cells were also transiently transfected with the pECE*kit* vector. NIH 3T3 cells do not express endogenous *c-kit*, and other investigators have previously shown that transfection of *c-kit* into NIH 3T3 cells resulted in an increase in the proliferation of the cells (Caruana *et al.*, 1998). In our hands, transient transfection of NIH 3T3 cells with murine *c-kit* did not result in any noticeable toxicity and, indeed, Figure 31B shows that there was a significant 50%

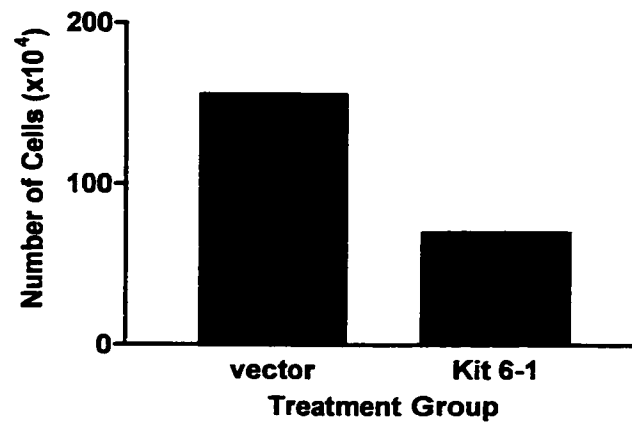
Figure 30

Inhibition of proliferation of A2780-cp ovarian carcinoma cells transfected with *c-kit* is lost upon freezing. Expression of *c-kit* mRNA in eleven different stably transfected A2780-cp clones was determined by northern blot analysis (A) using 15 µg of total RNA extracted from cultured cells and probing with a [³²P]-labeled human *c-KIT* cDNA. ACC-LC-80 human lung cancer cells and murine MC/9 mast cells were used as the positive controls for expression of the wild-type 5.1 kb mRNA (only 5 µg of the ACC-LC-80 RNA was loaded since these cells over-express *c-KIT*). LC-73 human lung cancer cells served as the negative control for expression of the human *c-KIT* mRNA, while a stable backbone vector-only clone (vector 1-2) was the negative control for expression of the murine 3.7 kb transfectant *c-kit* mRNA. All eleven of the *c-kit* stable clones shown here expressed the 3.7 kb transfectant *c-kit* cDNA. To assess the proliferation of the *c-kit*-transfected stable clones (Kit2-7, 5-1 and 6-1) relative to parental untransfected (parent) or backbone vector only-transfected cells (vector), the cells were cultured for 48 h and then counted using hemacytometry. Transfected cells, but not parental cells, were cultured in the presence of hygromycin. Although stable clone Kit6-1 showed an approximate 40% decrease in the rate of proliferation compared to backbone vector-transfected cells in one experiment (B), this difference in proliferation was no longer observed after the clones had been frozen and thawed (C).

A



B Pre-freezing



C Post-freezing

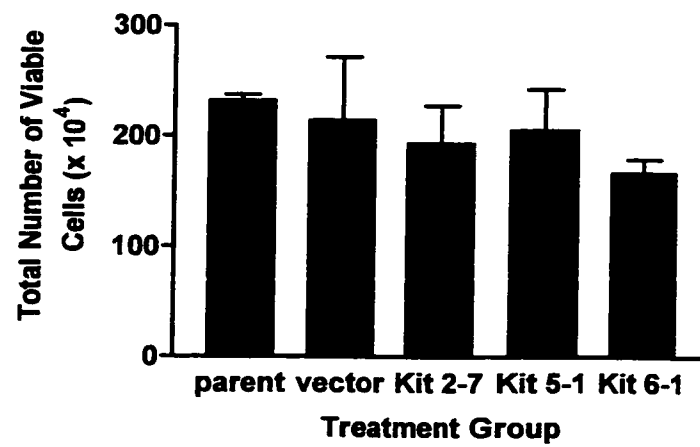
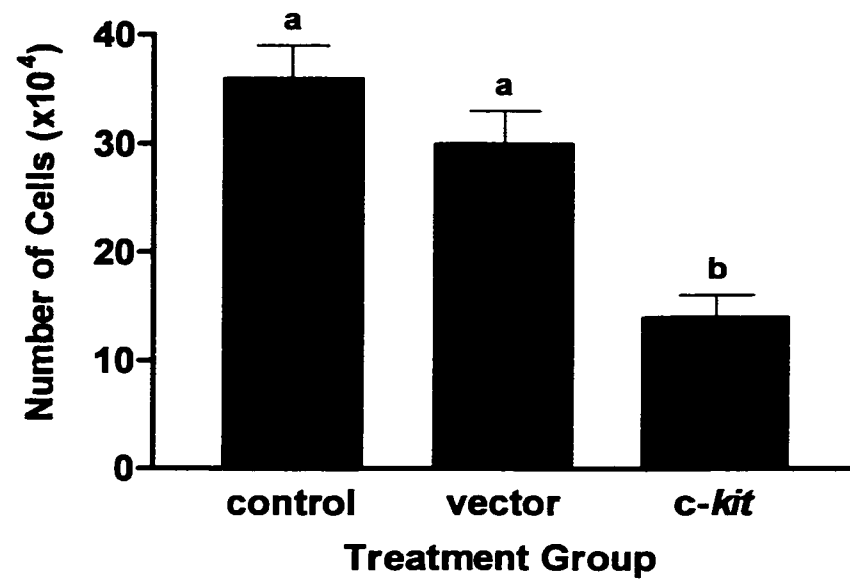


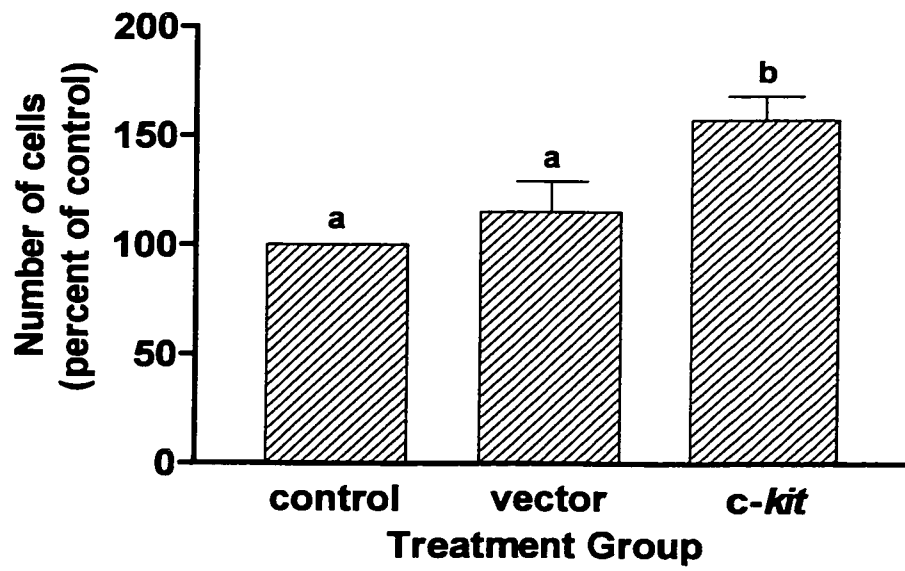
Figure 31

Transient expression of Kit protein causes decreased proliferation of HEY cells but not NIH 3T3 cells. An equal number of untransfected HEY or NIH 3T3 cells (control), or cells which were transiently transfected with the pECE backbone vector (vector) or pECE*kit* vector (*c-kit*), were cultured for 48 h, following which time the total number of cells was counted using hemacytometry. Values are the mean $\pm$ SEM from 3 experiments done in triplicate. Different lowercase letters above the bars indicates significant differences between treatment groups. *C-kit*-transfected HEY cells demonstrated a significantly decreased rate of proliferation compared to parental untransfected cells and backbone vector-transfected cells ($p<0.001$). In contrast, NIH 3T3 cells transfected with *c-kit* demonstrated a significant increase in proliferation relative to untransfected cells and backbone vector-transfected cells ($p<0.05$).

A) HEY



B) NIH 3T3



increase in the proliferation of the *c-kit*-transfected NIH 3T3 cells compared to either untransfected control cells or backbone vector-transfected cells ($p<0.05$).

5.4 Discussion

The results presented herein demonstrated that cytoplasmic KIT protein in HEY cells was tyrosine-phosphorylated and capable of *in vitro* autophosphorylation. Although an antisense oligonucleotide strategy was unable to knock out KIT expression in HEY cells, the converse experiment whereby HEY cells were transiently made to express additional *c-kit* resulted in growth suppression of these cells. Similarly, stable transfection of KIT-negative A2780-cp ovarian carcinoma cells with *c-kit* also resulted in decreased proliferation of those cells.

Since HEY cells co-express *c-KIT* and SCF, and HEY cells do not express KIT receptors at the cell surface, the finding that cytoplasmic KIT was tyrosine-phosphorylated in these cells suggests an intracellular SCF-mediated activation of KIT. Intracellular activation of another class III receptor tyrosine kinase, the PDGF receptor, has been shown in *v-sis* (PDGF)-transformed normal rat kidney cells (Keating and Williams, 1988). Additionally, Hines *et al.* (1995) reported that the growth advantage of *c-KIT*-transfected breast cancer cells over controls was only partially abrogated in the presence of neutralizing KIT antibodies, and they concluded that the predominant activation of KIT occurred through intracellular interactions with endogenous SCF. Alternatively, as described in Chapter 1, numerous studies have reported activating mutations in the *c-KIT* receptor that eliminate the

requirement for SCF-mediated activation, so we are currently sequencing HEY cell *c-KIT* to determine whether it contains any known activating mutations.

It is not known whether the inability to disrupt HEY cell KIT expression using antisense oligos in the current study was due to the cells not taking up the oligos or a failure of the oligos to effectively target the *c-KIT* mRNA transcripts. There is abundant evidence of the utility of antisense oligos in knocking out expression and function of growth-regulatory molecules in diverse cell types (Anfossi *et al.*, 1989; O'Keefe *et al.*, 1989; Broxmeyer *et al.*, 1991; Normanno *et al.*, 1996; Rubenstein *et al.*, 1996; Hayashi *et al.*, 1998; van Eylen *et al.*, 1998). The antisense oligos used in this study were the same as those employed by Ratajczak *et al.* (1992), who reported that these oligos not only blocked *c-KIT* mRNA expression in a dose-dependent manner but also inhibited growth of hematopoietic colonies from chronic myeloid leukemia and polycythemia vera patients. Other investigators have also demonstrated decreased colony-forming ability of bone marrow progenitor cells exposed to antisense *c-KIT* oligos (Catlett *et al.*, 1991). It would be worthwhile to repeat the HEY experiments using a transfected antisense *c-KIT* vector or an adenoviral approach instead of antisense oligos. For example, specific reduction in KIT protein expression and growth inhibition of small cell lung cancer cells was reported following their infection with adenovirus that expressed antisense *c-KIT* transcripts (Yamanishi *et al.*, 1996).

Transient transfection of HEY cells with murine *c-kit* caused significant growth suppression of those cells. Decreased proliferation following transfection of *c-KIT* has also been reported for a breast cancer cell line (Nishida *et al.*, 1996), a metastatic melanoma cell line (Huang *et al.*, 1996), as well as transformed fibroblasts and embryonal kidney cells

(Taniguchi *et al.*, 1999). Although HEY cells express SCF, decreased proliferation of HEY cells expressing murine *c-kit* is particularly remarkable since human SCF has been reported to be about 800-fold less active than murine SCF at activating murine Kit receptors (Martin *et al.*, 1990). This suggests, therefore, that the transfectant Kit protein in HEY cells may have some inherent ligand-independent growth-inhibiting ability. In support of this possibility, Caruana *et al.* (1998) reported that anchorage independent growth of some *c-KIT*-transfected NIH 3T3 cells could not be inhibited by neutralizing antibodies or SCF antisense mRNA.

Given that transient transfection of *c-kit* into HEY cells negatively regulated their proliferation, it is perhaps not surprising that it was very difficult to obtain cells that were stably expressing Kit. In initial stable transfection experiments, A2780-s and -cp cells that were transfected with *c-kit* were at a clear growth disadvantage in *in vitro* culture; indeed, no *c-kit*-expressing hygromycin-resistant colonies were recovered from those first experiments. Subsequently, however, a pooled population of *c-kit*-transfected A2780-cp cells containing 20-30% KIT-positive cells did demonstrate decreased proliferation compared with parental untransfected cells and pooled populations of backbone vector-transfected cells. Notably, the expression of *c-kit* in individual clones derived from the pooled *c-kit* transfectants appeared to be unstable upon freezing.

Transient transfection of A2780 ovarian carcinoma cells with the pKJ136 vector expressing human *c-KIT* resulted in a very different subcellular localization of KIT protein compared with the pECE*kit* vector that expressed murine *c-kit*. Although the reason(s) for this discrepancy is not known, perhaps the explanation lies in the inherent difference between

the two Kit sequences, i.e., the human *c-KIT* lacked the 12 bp juxtamembrane sequence while the murine *c-kit* sequence contained it.

It was important to verify that the decreased proliferation documented in HEY and A2780-cp cells transfected with *c-kit* did not simply reflect a toxicity of the *c-kit* vector. Therefore, NIH 3T3 fibroblast cells were also transfected with the pECE*kit* vector and their proliferative capability was assessed. In accordance with what had been previously reported (Lev *et al.*, 1990; Caruana *et al.*, 1998), transfection of NIH 3T3 cells with *c-kit* vector resulted in a significant increase in cellular proliferation. NIH 3T3 cells are known to produce murine SCF (Jozaki *et al.*, 1991), and the 50% increase in proliferation observed in the current study probably reflects the interaction of endogenous SCF with the transfectant Kit. However, since the subcellular localization of the transfectant Kit in the NIH 3T3 cells was not examined here, the subcellular site of this Kit:SCF interaction is not known. Therefore, it is possible that a greater increase in proliferation would be observed if the *c-kit*-transfected NIH 3T3 cells were cultured in serum-free media with exogenous murine SCF. The ability of *c-kit* expressed from the pECE*kit* vector to decrease proliferation in ovarian carcinoma cells but increase proliferation in normal fibroblast cells suggests that different signalling pathways are being activated in normal versus cancer cells, or in epithelial versus fibroblast cells, and this is currently being addressed in our laboratory.

Thus, the finding that enforced expression of *c-kit* in human ovarian carcinoma cells negatively regulates their growth suggests a mechanism underlying the positive prognostic value of *c-KIT* expression in malignant epithelial ovarian tumors. Whether activated KIT can also affect the chemosensitivity of ovarian cancer cells is addressed in Chapter 6.

Chapter 6 - Concluding Remarks

This Ph.D. project sought to determine the expression, regulation, and function of the KIT tyrosine kinase receptor and its ligand, SCF, in human epithelial ovarian cancer. Normal human OSE cells did not express *c-KIT* *in vitro* or *in vivo* but did express SCF, and OSE invaginations and inclusion cysts frequently expressed *c-KIT*. Greater than 70% of the epithelial ovarian cancers examined expressed *c-KIT*, and almost all of these co-expressed SCF. There was a positive clinical correlation between *c-KIT* expression in malignant epithelial tumors and disease-free patient survival, suggesting that an autocrine KIT:SCF interaction may negatively regulate ovarian cancer cell growth. Factors which regulated the proliferation of ovarian carcinoma cells, such as TGF- α and dbcAMP, also regulated the expression of *c-KIT*, providing further evidence to suggest a role for *c-KIT* in regulating ovarian cancer cell proliferation. Indeed, enforced expression of *c-kit* in ovarian carcinoma cells, as compared with normal fibroblast cells, resulted in a cell type-specific suppression of proliferation.

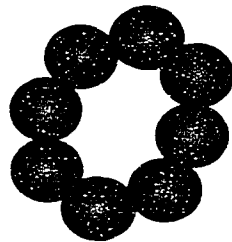
Based on the findings of this Ph.D. project, therefore, the following model is proposed to explain the role of the KIT:SCF interaction in human epithelial ovarian tumorigenesis (Figure 32). Expression of *c-KIT* in about 50% of OSE-lined inclusion cysts, coupled with the finding that enforced KIT expression caused a decrease in ovarian carcinoma cell proliferation, suggests that activated KIT may suppress the growth of ovarian neoplasms arising from KIT-positive inclusion cysts, thereby rendering a less aggressive tumor phenotype. This is supported by the data presented herein showing that a larger

Figure 32

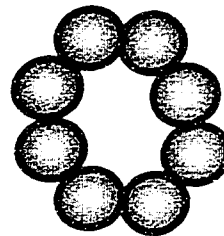
A model of the role of the KIT:SCF interaction in human epithelial ovarian tumorigenesis. The results presented in this thesis demonstrated that women whose malignant tumors expressed *c-KIT* had a better prognosis, and enforced *c-KIT* expression in ovarian carcinoma cells suppressed their proliferation. Expression of *c-KIT* in about 50% of OSE-lined inclusion cysts, coupled with the growth-suppressive role of activated KIT in ovarian carcinoma cells, suggests that ovarian neoplasms arising from *c-KIT*-positive inclusion cysts would have a less aggressive tumor phenotype. Expression of the KIT:SCF system in malignant tumors would also function to decrease tumor cell proliferation, suggesting an explanation for the better prognosis for women with *c-KIT*-positive tumors. Thus, the existence of an autocrine growth-suppressive KIT:SCF system may represent a fundamental target for dysfunction during tumor progression in advanced stage disease. Ideally, detection of elevated levels of soluble KIT in patients' blood could be used as a diagnostic and/or prognostic biomarker.

normal OSE  **KIT⁻/ SCF⁺**

**inclusion
cyst**



KIT⁻

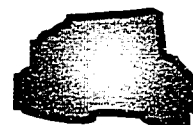


KIT⁺

**ovarian
tumor**



KIT⁻/ SCF⁺



↓ growth

KIT⁺/ SCF⁺

more aggressive

less aggressive

worse prognosis

better prognosis

proportion of benign and LMP tumors expressed c-*KIT* compared with malignant tumors. (Although the impressive size achieved by some benign tumors might appear to argue against the importance of a growth-inhibitory KIT:SCF function, in fact these large tumors do not rapidly proliferate and, furthermore, they are usually comprised of a high stromal:epithelial ratio and/or are highly cystic in nature rather than possessing hyper-proliferating epithelium.) This model is further supported by the finding that women whose malignant tumors did not express c-*KIT* had a worse prognosis, suggesting that activated KIT also negatively regulates the growth of malignant tumor cells *in vivo* as was demonstrated for ovarian carcinoma cells *in vitro*. Thus, the lack of a functional KIT:SCF system may contribute to malignant transformation of OSE cells, while the existence of an autocrine growth-suppressive KIT:SCF signalling loop may represent a fundamental target for dysfunction during tumor progression in advanced stage disease. Anecdotal support for this model comes from studies showing that viable mutant mice that lack functional KIT develop complex epithelial ovarian neoplasms shortly after birth (Murphy, 1972; Kissel *et al.*, 2000).

The model presupposes that the KIT:SCF interaction in normal OSE cells would function to negatively regulate proliferation. However, both of the ovarian carcinoma cell lines that demonstrated decreased proliferation upon transfection with c-*kit* in this project, HEY and A2780-cp, are transformed cell lines that will form tumors in nude mice and, therefore, they are more representative of advanced stage epithelial ovarian cancer than early stage disease. The finding that normal fibroblasts were growth-stimulated when transfected with c-*kit* might predict that c-*KIT* expression in another normal cell type, OSE cells, may stimulate, rather than inhibit, their proliferation and the model would then become more

complicated.

There are a number of experiments that could be carried out to ascertain the function of KIT in early epithelial ovarian tumorigenesis. For instance, a method for microdissecting human OSE-lined inclusion cysts from frozen normal ovaries has recently been published (To *et al.*, 1998), so it would be interesting to try to establish these cells in culture and compare the *in vitro* growth and survival of c-*KIT*-negative versus c-*KIT*-positive OSE cells. The penultimate experiment to prove this model would be to transfect normal human OSE cells with c-*KIT* to determine whether activated KIT can influence the proliferation of these cells. Our laboratory has recently obtained two immortalized human ovarian surface epithelial cell lines, HIO-117 and HIO-135 (generously provided by Dr. A. Godwin, Philadelphia, PA), and these, as well as primary cultures of normal OSE cells, could be transfected (or infected) with c-*KIT*. The model described above would predict that expression of c-*KIT* in these cells should decrease their proliferation. If, however, the converse occurs and the proliferation of normal OSE cells actually increases with aberrant KIT expression, as was observed when normal fibroblast cells were transfected with c-*kit*, then additional endpoints such as colony formation in soft agar and tumor formation in nude mice could be assessed.

In addition to the aforementioned *in vitro* experiments, injecting adenovirus expressing c-*kit* under the ovarian bursa of mice to try to infect the OSE cells *in situ* could also be attempted. The bursa is a transparent sheath that encloses rodent ovaries, so injecting under the bursa should allow for the OSE cells to be bathed in, and infected by, c-*kit*-expressing adenovirus. Dr. B. Vanderhyden has successfully infected mouse OSE cells with

a retrovirus expressing *lacZ* using this approach.

The results of this thesis demonstrated that c-*KIT* expression in epithelial ovarian tumors has the potential to be an independent prognostic variable. However, it is necessary to determine the c-*KIT* expression in more tumor specimens, particularly from stage IV disease, and confirm that the statistical correlation with patient survival is upheld. In the time period since the study in Chapter 3 was completed, greater than 100 additional tumor specimens have been accumulated. Although no significant correlation between c-*KIT* expression and tumor grade/degree of differentiation was found in this study, the documented decrease in, or absence of, c-*KIT* expression in the high grade, advanced stage tumors suggests the possibility that KIT expression is contributing to a more differentiated, less aggressive tumor phenotype. Screening of additional tumors should also shed some light on this possibility. Furthermore, whether the measurement of soluble KIT in patients' serum could be used as a diagnostic or prognostic variable in epithelial ovarian cancer remains to be determined. There are commercially available kits for the measurement of s-KIT, and determination of whether primary cultures of ovarian cancer cells produce s-KIT would be carried out prior to acquiring and testing patients' blood samples.

Finally, the correlation between c-*KIT* expression and longer patient survival also suggests another hypothesis, that KIT may increase the chemosensitivity of ovarian cancer cells. Indeed, it was with this possibility in mind that the c-*kit*-negative A2780-s and -cp cells, which are cisplatin-sensitive and cisplatin-resistant, respectively, were selected for c-*kit* transfection studies. Experiments ongoing in our laboratory are treating untransfected and transiently c-*kit*-transfected A2780-s and -cp cells with cisplatin or taxol, and the ability of

Kit to modulate cisplatin toxicity is being assessed by counting viable and dead cells and comparing untransfected versus *c-kit*-transfected cells. Thus, these studies may identify an additional physiological role of *c-KIT* in ovarian tumorigenesis.

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