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THE EFFECTS OF HORMONES, GROWTH FACTORS AND CYCLIC AMP ON OVARIAN CARCINOMA CELL PROLIFERATION AND EXPRESSION OF C-*KIT* AND KIT LIGAND

by

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Thesis submitted to the School of Graduate Studies and Research
at the University of Ottawa in partial fulfillment
of the requirements for the degree of

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DEDICATION

There are many people who have had a profound influence in the shaping of my life, both academically and personally, and it is to all of you that I dedicate this work.

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JOE HIKAN SHIN SUI SHO GO -

" Embrace hard things and your mind will blossom"

(Japanese proverb)

ABSTRACT

The protooncongene c-*kit* encodes a tyrosine kinase receptor (Kit) that is activated by its ligand KL to promote proliferation, survival and migration of Kit-bearing cells. In addition to its ability to induce transformation in transfected cells, c-*kit* is overexpressed in many cancers, including those of the ovary, suggesting a strong oncogenic capability for this growth factor receptor. C-*kit* and KL gene expression have also been shown to be differentially regulated by various growth factors and hormones. Therefore the goal of this research project was to examine the regulation of c-*kit* and KL expression in ovarian carcinoma cells, by factors which are produced by, and/or act on the ovary, such as steroid and gonadotropic hormones, and growth factors.

Studies were completed using two ovarian carcinoma cell lines, HEY and OVCA 429, which are both c-*kit*- and KL-bearing, as *in vitro* model systems for ovarian carcinoma. Using these 2 cell lines, the effects on proliferation and c-*kit* and KL mRNA expression of: epidermal growth factor (EGF), transforming growth factors-α and β (TGF-α and TGF-β), estradiol (E_2), progesterone (P_4), testosterone (T), follicle stimulating hormone (FSH), human chorionic gonadotropin (hCG), cyclic adenosine monophosphate (cAMP) analogues (dibutyryl-adenosine-3':5'- monophosphate, cyclic (dbcAMP) and 8-bromo-adenosine-3':5'-monophosphate, cyclic (8-bromo-cAMP)) and an adenylate cyclase activator (cholera toxin) were determined. HEY cells were not viable in serum-free media, and culture of these cells in the presence of growth factors (EGF, TGF-α or TGF-β) under varying serum conditions did not alter their proliferation rate or expression of c-*kit*/KL transcripts. Furthermore, after

48 h, none of the exogenously applied hormones (E_2, P_4, T, FSH or hCG) exerted any significant effects on cell proliferation or gene expression in both HEY and OVCA 429 cells. However, using three distinct membrane-permeable molecules, direct stimulation of the downstream cAMP signalling pathway for gonadotropin receptors was achieved. Treatment of HEY cells with dibutyryl cyclic AMP (dbcAMP) over a range of concentrations from 0.25 - 2.0 mM caused significant dose-dependent inhibition of HEY cell proliferation by up to 40% ($p<0.001$ at 2 mM). Significant inhibition of proliferation was reproducibly induced by two other molecules which elevate intracellular cAMP; 8-bromo-cAMP (62% inhibition with 2 mM; $p<0.001$) and cholera toxin (CT; 38% inhibition at 1.0 μg/ml). At the transcriptional level, northern analysis demonstrated that both dbcAMP and CT produced marked elevations in c-*kit* mRNA expression (up to 5- and 7-fold greater relative to control, respectively), which inversely correlated in a dose-dependent manner with the observed decreases in proliferation. There were no corresponding changes in the levels of KL mRNA expression. Although baseline levels of Kit protein expression were weakly detectable in untreated HEY cells, addition of 2mM dbcAMP resulted in concomitant increases in Kit protein levels. Furthermore, dbcAMP-induced inhibition of HEY cell proliferation was not reversed by addition of an anti-Kit neutralizing antibody. Taken together, these results would indicate that while inhibition of ovarian carcinoma cell proliferation by cAMP stimulation is associated with upregulation of c-*kit* mRNA and protein expression, Kit is not directly linked to the cAMP-mediated pathway of proliferation regulation in HEY cells.

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LIST OF ABBREVIATIONS

ATP	adenosine triphosphate
8-bromo-cAMP	8-bromo-adenosine-3':5'-monophosphate, cyclic
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CSF	colony stimulating factor
CT	cholera toxin
dbcAMP	dibutyryl-adenosine- 3':5'-monophosphate, cyclic
DNA	deoxyribonucleic acid
DEPC	diethylpyrocarbonate
DMEM	Dulbecco's modified essential medium
DTT	dithiothreitol
DMSO	dimethylsulfoxide
E_2	estradiol-17β
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
FCS	fetal calf serum
FITC	fluorescein
FSH	follicle stimulating hormone
FSH-R	follicle stimulating hormone receptor
G_s protein	G protein; stimulatory
h	hour(s)
hCG	human chorionic gonadotropin
HRP	horseradish peroxidase
IgG	immunoglobulin
IL-1	interleukin-1
IL-6	interleukin-6
ITS	insulin-transferrin-selenium

kD	kilodalton
KL	Kit ligand
LH	luteinizing hormone
LH-R	luteinizing hormone receptor
LHRH	luteinizing hormone-releasing hormone
mAB	monoclonal antibody
MAP	mitogen activated protein kinase
MEM	mimimal essential medium
ml	millilitre(s)
min	minutes
mRNA	messenger ribonucleic acid
NEAA	non-essential amino acids solution
OSE	ovarian surface epithelium
P_4	progesterone
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PKC	protein kinase C
PMSG	pregnant mares' serum gonadotropin
PTP	protein tyrosine phosphatase
ROSE	rat ovarian surface epithelium
RT	room temperature
RTK	receptor tyrosine kinase
RT-PCR	reverse transcription-polymerase chain reaction
SH-2	*src* homology domain-2
Sl	Steel locus
S-PBS	Stockholm's phosphate buffered saline
SDS	sodium dodecyl sulfate
SSC	saline sodium citrate
T	testosterone
TAA	tumour associated antigen

TCA	trichloroacetic acid
TGFα	transforming growth factor-alpha
TGFβ	transforming growth factor-beta
UV	ultraviolet light
W	*White* spotting locus

INTRODUCTION

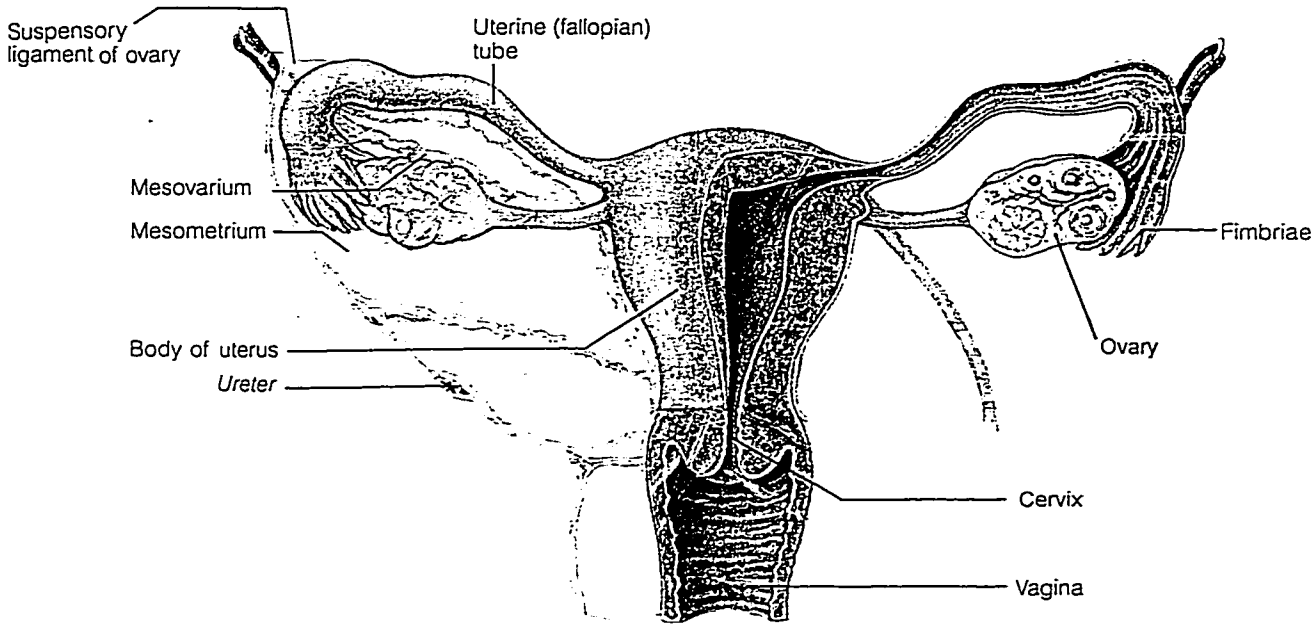
1. Ovarian Cancer

1.1 Basic Features of the Ovary

The paired ovaries are the main endocrine glands of the human female reproductive system. In addition, the ovaries are the site of formation and release of the female germ cells (oocytes), by a process called oogenesis. The ovaries are almond-shaped, pinkish-white structures which flank the uterus bilaterally, and measure about 3-5 cm long, 1.5 cm wide, and 1 cm thick. They are located near the fimbriae of the uterine tubes, and are held in place by peritoneal mesenteries and ligaments (Figure 1). Before puberty, the surface of the ovary is smooth, but thereafter it becomes progressively scarred and pitted due to repeated ovulations. At puberty, the ovaries are activated by pituitary gonadotropins (FSH and LH) to develop the ovarian follicles and to secrete sex hormones (estrogen, progesterone and testosterone) (Anderson, 1985; Marieb & Mallett, 1992; Moore & Persaud, 1993).

The outer surface of the ovary is covered by a layer of simple cuboidal epithelium inappropriately known as the germinal epithelium. This surface epithelium is embryologically derived from the mesodermal epithelium of the genital ridges, and is continuous with the flattened mesothelium of the peritoneum forming the mesovarium (Moore, 1992). As the fetus develops, primordial germ cells which migrated to the genital ridges (also known as the gonadal or germinal ridges) from the yolk sac become incorporated into primordial follicles and migrate into the ovarian cortex. Each primordial follicle consists of an oocyte derived from a primordial germ cell surrounded by a single layer of flattened follicular cells derived from cortical cords, extensions of the ovarian surface

Figure 1: ***Posterior view of the ovaries and related structures.*** The broad ligament is removed on the right side. Each ovary is connected/supported by three ligaments: (1) to the posterior border of the broad ligament by the mesovarium, (2) by the ovarian suspensory ligament to the lateral wall of the pelvis and (3) to the lateral angle of the uterus by the fibrous ligament of the ovary (Adapted from Marieb & Mallatt, 1992).


Suspensory ligament of ovary
Uterine (fallopian) tube
Mesovarium
Mesometrium
Fimbriae
Body of uterus
Ureter
Ovary
Cervix
Vagina

epithelium (OSE) (Moore & Persaud, 1992; Marieb & Mallatt, 1992). A number of marked changes in OSE morphology occur during the fourth and fifth months of gestation, as the surface epithelium undergoes intense proliferation and invades the stroma to form the cortical cords. Using light and electron microscopy, Gondos (1975) studied the features of the OSE during this time, and determined a number of proliferative characteristics reminiscent of malignancy. These included disruptions of cellular orientation, prominent nucleoli, nuclear infolding and distortion, irregular chromatin distribution, loss of epithelial polarity and decreased numbers of desmosomes. By the 24th week, this dramatic activity ceases, and the OSE reverts to its mature single-cell surface layer.

The single-cell layer of adult OSE is separated from the ovarian cortex (and tunica albuginea) by an underlying basement membrane, and can range phenotypically from simple squamous, to cuboidal, to low columnar pseudostratified epithelium (reviewed in Murdoch, 1996). OSE cells are characterized by numerous desmosomes, gap junctions, tight junctions and apical microvilli; several endocytic vesicles (for fluid absorption) are contained within the cytoplasm (Perez *et al.*, 1991; Auersperg *et al.*, 1994).

Unlike most epithelial cells, OSE cells behave like generative stem cells, that with each division produce *two* daughter cells with continued growth potential; thereby remaining uncommitted and pluripotent (Dyck *et al.*, 1996; Hamilton, 1992). OSE cells are characteristically known for their morphologic plasticity i.e., *in vivo*, they are cuboidal (usually attain a cobblestone-like appearance), while *in vitro* they usually become flatter and resemble more atypical, fibroblastic-like cells (Auersperg *et al.*, 1984; Siemens & Auersperg, 1988). Furthermore, OSE cells produce extracellular matrix components characteristic of

both epithelial and stromal (mesenchymal) cells (Auersperg *et al.*, 1994), suggesting a means for epithelio-fibroblastic-like plasticity.

1.2 Incidence Rates and Early Screening

Ovarian cancer is the leading cause of death of all gynecologic malignancies, and the fifth leading cause of cancer-related death in women (5%) in the United States (Landis *et al.*, 1998). According to data from the National Cancer Institute's Surveillance, Epidemiology, and End Results (SEER) program in the U.S., it is estimated that 25,400 new cases will be diagnosed in 1998, and 14,500 deaths will occur from ovarian cancer (Landis *et al.*, 1998). In more appreciable terms, approximately 1 in 70 women (1.4%) will develop ovarian cancer during their lifetime (Dunnihoo, 1992). Estimates suggest that the five-year survival rate from ovarian cancer is 93 percent, provided it is diagnosed and treated early, when it is still localized to Stage I (Landis *et al.*, 1998). Unfortunately, cancer of the ovary is relatively asymptomatic, and therefore most ovarian cancers are not recognized early; in fact only 24 percent of all cases are detected at the localized stage (Landis *et al.*, 1998). While some patients may complain of vague gastrointestinal disturbances, discomfort, abdominal swelling (ascites), or abnormal vaginal bleeding (rarely), early-stage ovarian cancer is generally without warning signs. This small, almond-shaped organ may grow to considerable size before clinical symptoms appear. Even with aggressive treatment, patients with advanced-stage ovarian cancer face a dismal prognosis: only 15-25 percent survive and are disease-free after 5 years (Teneriello & Park, 1995; Landis *et al.*, 1998). Therefore, a more realistic five-year survival rate for all stages combined is 47 percent; nearly half of the early-

diagnosed survival rate (Landis *et al.*, 1998).

Currently there are no effective screening tests for ovarian cancer. Historically, the family history and physical examinations were the only techniques available for early detection of ovarian cancer. Approximately 5% of ovarian cancers are familial in nature with a discernible pattern of heredity (Teneriello & Park, 1995). Considerable attention has been focused on allelic losses from chromosome 17, which includes the BRCA-1 locus (Eccles *et al.*, 1990; Jacobs *et al.*, 1993). Mutations to the BRCA-1 tumour suppressor gene are associated at high frequency with familial ovarian cancer syndromes such as the breast/ovarian cancer syndrome (Eccles *et al.*, 1990; Jacobs *et al.*, 1993). In addition, several screening tools have become available and are used with varying frequency and usefulness. These include ultrasonography (transabdominal and transvaginal), colour flow Doppler imaging, magnetic resonance imaging, positron-emission tomography, and pelvic and abdominal computed tomography (Teneriello & Park, 1995). While each of these methods offers something in addition to the traditional pelvic exam, none of these tools has demonstrated its usefulness as a mass screening detection modality and, furthermore, there is great variation in their sensitivity and/or specifity for ovarian cancer.

In recent years, considerable attention has been paid to the emergence of tumour-specific biomarkers as readily assessed detectors of disease. These so-called Tumour-Associated Antigens (TAAs) are serum markers present on, or released by, tumour cells which are not produced by normal cells, and can be assayed using monoclonal antibodies. Recognized biomarkers in ovarian cancer include CA-125, TAG-72, OVX1, M-CSF and CA 15-3 (reviewed in Teneriello & Park, 1995). These TAA's have shown some promise as

indicators of disease status, progression and recurrence; however their efficacy in the screening detection of ovarian cancer has been limited (Teneriello & Park, 1995). Unfortunately, many, if not all, of the TAA markers identified thus far are not unique to cancer of the ovary, and may exhibit cross-reactivity to other cancers. For example, elevations in CA-125 have been found in patients with breast, cervical, lung and pancreatic cancer (Niloff, 1992), and increased levels have also been associated with benign conditions such as endometriosis or even pregnancy (Teneriello & Park, 1995). Additionally, not all types or stages of ovarian neoplasms are associated with elevated levels, and therefore the usefulness of TAA's in ovarian cancer screening is lessened.

1.3 Classification of Ovarian Tumours

Primary ovarian tumours are classified according to the ovarian structure from which the cancer is derived. Despite composing only a small fraction of the total ovarian mass, nearly 90% of all ovarian cancers arise from the surface epithelium (Woodruff, 1976; Weiss *et al*, 1977; reviewed in Anderson, 1985; Murdoch, 1996; Young *et al*, 1989). Nonepithelial ovarian malignancies are less common, and account for the remaining 10% of all tumours. The most predominant of the non-epithelial ovarian tumours are those derived from germ cells, but this category also includes tumours of sex cord-stromal origin, and extremely rare malignant cancers such as lipoid cell tumours and mixed mesodermal sarcomas (Berek *et al.*, 1993).

There are four basic stages of disease progression in ovarian cancer as assessed by the International Federation of Gynecology and Obstetrics (FIGO). FIGO Stage I is

classified as cancer that is found only in one (Stage Ia) or both (Ib) of the ovaries, and is further defined by the presence of epithelial inclusion cysts which invade the ovarian cortex. Prognosis is good if the growth has remained limited internally (Ia or Ib), 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). Stage II disease, involving one or both ovaries, shows extension of malignant cells into the pelvic cavity (IIa, oviducts/uterus; IIb, other pelvic tissues) and/or ascites (IIc). The spread of peritoneal implants within the abdomen outside of the pelvis (IIIa, microscopic seeding; IIIb and IIIc, macroscopic nodules $\leq$ or $\geq$ 2 cm in diameter) with or without surrounding lymph nodes involvement (also IIIc, retroperitoneal or inguinal nodes), is the hallmark of Stage III disease. This highly invasive stage may include extension of cancerous cells onto the surface of the liver or intestine. The highly metastatic Stage IV is also found in one or both ovaries, but with extensive disseminated metastases outside of the abdomen or beyond the liver surface (reviewed in Hamilton, 1992; Murdoch, 1996; Rees, 1995).

Histological classification of ovarian carcinomas is made on the basis of similarities to normal genital and urologic tissues: (1) serous = fallopian tube; (2) mucinous = endocervix; (3) endometrioid = endometrium; (4) celioblastomas (also known as Brenner tumours) = transitional; (5) clear cell carcinomas = mesonephric; (6) mixed epithelial; (7) undifferentiated; and (8) unclassified carcinomas (Czernobilsky, 1987). Almost fifty percent of all ovarian carcinomas are of serous histopathology (Ozols *et al.*, 1992), with the other cell types making up the remainder. Ovarian tumours are further categorized on the basis of their metastatic potential into benign, borderline/low malignant potential or malignant neoplasms.

The latter are histologically graded into three subcategories: differentiated (Grade I), moderately differentiated (Grade II), or poorly differentiated (Grade III), which describe how closely the tumour cells resemble their normal counterparts, and attempt to offer some prognostic information from an estimate of the tumour's growth rate (Pfeifer & Wick, 1995).

1.4 Proposed Etiology and Risk Factors

Ovarian cancer is a highly deadly and insidious disease which predominantly affects post-menopausal women. The etiology of this disease is unknown, although a number of theories have been described. Almost 90% of all ovarian cancers are believed to arise from the single layer of epithelial cells that surround the ovary. The most prevalent theories implicate trauma to the ovarian surface arising from one of two mechanisms (incessant ovulation or inclusion cyst formation) that induce disturbances in OSE growth that lead to tumour formation.

Following ovulation, rapid proliferation of epithelial cells occurs at wound edges in order to repair the rupture site on the ovarian surface (reviewed in Godwin *et al.*, 1992a; Murdoch, 1996). It is believed that OSE cells in the area of the rupture site(s) are actively involved in the process of follicular rupture, by producing and excreting large enzyme-containing lysosomal bodies which degrade the tunica albuginea and underlying follicular cells (Bjersing & Cajander, 1974). In 1971, Fathalla initially proposed the "incessant ovulation" theory, suggesting that continuous ovulation causes repeated trauma to the ovary which leads to ovarian cancer development. He had attempted to explain the protective effect of multiple pregnancies, and noted that the number of ovulations during a woman's

lifetime (which are interrupted during pregnancy), was directly correlated to increased risk (Fathalla, 1971). The repetitive proliferation of these cells may increase the frequency of spontaneous mutations, which could conceivably be passed on to subsequent generations of cells. One piece of cited evidence for this repetitious growth hypothesis is derived from the malignant transforming potential of rat OSE cells which have been continuously subcultured to mimic incessant ovulation. Following repeated passages, these cells attain features associated with transformation such as loss of contact inhibition, substrate-independent growth, and the ability to form tumours in nude mice (Godwin *et al.*, 1992b). Conversely, the "excess gonadotropin secretion theory" suggests that uninterrupted ovulation may predispose to ovarian cancer by increasing exposure to high levels of gonadotropins, which may themselves be responsible for tumourigenesis (Cramer & Welch, 1983).

Surface epithelial inclusion cysts are believed to arise from the invagination and trapping of the ovarian surface, which becomes sealed off and allows small cysts to form in the ovarian stroma. This may occur during remodeling of the ovarian surface following ovulation-induced proliferation or follicular attrition, or alternatively from inflammation caused by carcinogens or chemical irritants (such as asbestos, talc) (Hamilton, 1992; Scully, 1993). OSE-lined inclusion cysts and crypts are common in women, and generally benign in nature, although they are widely held to be the origin of many epithelial cancers, following early malignant changes (Young *et al.*, 1989).

Until recently, there was considerable debate over the clonality of this particular disease, with some suggestion that ovarian cancer was a multifocal, and not a unifocal disease arising from a single cell (first reported by Woodruff & Julian, 1969). If this were

the case, it was postulated that multiple peritoneal tumours were primaries which arose independently, and not simply distant metastases from a single ovarian cancer. However, analysis of clonality in ovarian tumour samplings by current molecular techniques (p53 mutations, X chromosome inactivation and loss-of-heterozygosity measurements) provided direct evidence that most (92%) ovarian epithelial cancers were monoclonal in origin (Jacobs *et al.*, 1992; Mok *et al.*, 1992).

Arguments have also been put forth to suggest that ovarian carcinomas may develop from Müllerian remnants within paraovarian tissues, based on the resemblance of ovarian epithelial tumours to those arising from Müllerian-derived structures (McCluskey & Dubeau, 1997). It must also be noted, however, that the common derivative of the surface epithelium and the Müllerian-derived structures (fallopian tubes, cervix, and uterine body) to which these ovarian tumours have been compared, are adjacent sites in the embryonic coelomic epithelium. Therefore, the histological similarities between the two structures may be explained by their common embryological origin (Czernobilsky, 1987).

There are several risk factors which have been identified as predisposing to ovarian cancer development. Long menstrual intervals i.e., early menarche and late menopause, uninterrupted or hyper-ovulation (nulliparity and infertility drugs respectively), high fat diets, use of talc (presumably asbestos-contaminated), North American or European descent, genetic involvement (i.e., genetic anomalies or positive family history) and age, have all been correlated to greater individual risk of ovarian cancer (Bast *et al.*, 1995; Berchuck, 1995; Berek *et al.*, 1993; Cramer *et al.*, 1983a/b; Murdoch, 1996). Conversely, protective effects against ovarian cancer development have been associated with multiple pregnancies,

prolonged lactation, tubal ligation and the use of oral contraceptives (Hankinson, 1993; NIH Statement, 1994).

1.5 Incidence: Relation to Menopause

It is well established that the incidence of ovarian cancer increases around the perimenopausal period, when the serum levels of pituitary gonadotropins are elevated, and those of ovarian steroids (eg. estradiol) decline substantially (Longo & Young, 1981; Reyes *et al.*, 1977; Lee *et al.*, 1988). In fact, the median age for epithelial ovarian cancer is 60-65 years, with only 10-15% of tumours discovered in pre-menopausal women (Sell *et al.*, 1990). Not surprisingly then, ovarian regulatory hormones which are excessively secreted at the time of menopause have been suggested to play a role in ovarian carcinogenesis (Gardner *et al.*, 1961, Stadel *et al.*, 1975). As early as 1983, Cramer and Welch had suggested that excessive and continued gonadotropin secretion, as seen during menopause, contributed to malignant transformation by exerting trophic effects on OSE cells (Cramer & Welch, 1983). In particular, measurement of hormonal levels in women of varying age groups has indicated that gonadotropin levels, and particularly those of FSH, begin rising several years prior to the menopause (Reyes *et al.*, 1977). Elevations in gonadotropic levels are related to the significant decrease in follicle numbers within the ovary caused by a lifetime of steady logarithmic decline, and the substantial rise in gonadotropins may further be attributable to an age-related decrease in sensitivity at the level of the hypothalamus-pituitary, to feedback inhibition normally mediated by circulating estrogens (Reyes *et al.*, 1977; reviewed in Richardson, 1993).

Gonadotropin involvement in ovarian cancer has been demonstrated by the stimulatory effects of FSH and LH on human epithelial ovarian cancer cell proliferation *in vitro* (Feng *et al.*, 1996; Simon *et al.*, 1983; Wimalasena *et al.*, 1993), *in vivo* (Schiffenbauer *et al.*, 1997; Kurbacher *et al.*, 1995; Wimalasena *et al.*, 1992), and in promoting angiogenesis of transplanted ovarian carcinoma *in vivo* (Schiffenbauer *et al.*, 1997). Additionally, growth of human ovarian carcinomas in nude mice was inhibited following addition of an antagonist to the hypothalamic hormone, luteinizing hormone-releasing hormone (LH-RH) (Yano *et al.*, 1994). Gonadotropin receptors (FSH-R and LH-R) have been shown to be expressed on a number of solid ovarian tumour cells (Stouffer, 1984; Kammerman *et al.*, 1981; Kobayashi *et al.*, 1996; Mandai *et al.*, 1997), and on ovarian carcinoma cell lines *in vitro* (Hamilton *et al.*, 1983; Mandai *et al.*, 1997), and therefore the potential for gonadotropin involvement in the growth and transformation of OSE remains strong. Furthermore, the involvement of gonadotropic hormones in ovarian cancer has been supported by the protective effects of oral contraceptives, multiple pregnancies, and estrogen replacement therapy, all of which reduce the exposure of OSE cells to gonadotropins (reviewed in Gross & Schlesselman, 1994). Use of oral contraceptives for as long as five years decreases the risk of ovarian cancer by 50%, and even short-term use has been deemed to offer greater protection than absence of use (Stanford, 1991; Gross & Schlesselman, 1994).

2. Oncogenes and Protooncogenes

2.1 Definitions and General Role in Cancer

It is unlikely that alteration or defect of a single gene is responsible for cancer (Berchuck *et al.*, 1993). Instead, cancer probably results from multiple events which upset the dynamic balance of two important classes of genes: oncogenes and tumour suppressor genes (Boveri, 1929; Bishop, 1991; Hunter, 1991; Sager, 1989). Both protooncogenes and tumour suppressor genes are intricately involved in normal cellular growth and differentiation processes (Bishop, 1991; DiCioccio & Piver, 1992; Sager, 1989; Weinberg, 1988). Oncogenes are the prototypical cancer-causing genes: they are homologous to normal cellular genes (protooncogenes), and only require the genetic alteration of one allele to transform cells. They were first discovered in retroviruses, and are thought to have been acquired from animal hosts which became activated to possess transforming capabilities (reviewed in Berchuck *et al.*, 1992a). These dominant transforming genes are normally part of growth stimulatory pathways, and their activation by mutation, deletion, overexpression or translocation is oncogenic. Tumour suppressor genes (antioncogenes) are regulatory *inhibitors* of cell growth, and when mutated, they lose their ability to suppress tumour growth. Therefore, inactivation or loss of *both* tumour suppressor gene alleles is required for cell transformation (recessive transforming genes) (Berchuck *et al.*, 1992a). Well-known tumour suppressors include p53 and retinoblastoma (Rb), which both function at 'checkpoints' in the cell cycle to suppress unrestrained growth (promote apoptosis, programmed cell death). Additionally, the intact genes appear to be critical in the repair of DNA damage, and loss-of-function of these genes enables damaged cells to attain a

malignant phenotype and the potential for unrestrained growth (Barnes *et al.*, 1997).

Protooncogenes were originally identified as the normal cellular cousins of oncogenic transforming viruses, and have important functions of their own in some aspect of cell division or proliferation. It is now understood that protooncogenes only *resemble* retroviral oncogenes (by amplification, structural alteration or coding sequence), and not all protoncogenes are ultimately fated to undergo a cancer-causing mutational event. However, it is this functional feature of protooncogenes which confers upon them oncogenic capabilities *if* they become activated by a mutational event.

2.2 Receptor Tyrosine Kinases

More than 100 oncogenes have been classified on the basis of the functions of their homologous protooncogene products. Various classification schemes have been documented, which may be summarized as follows: (1) growth factors (eg. *sis, hst*); (2) receptor tyrosine kinases (also classified as growth factor receptors; eg. *erb*B, *neu, fms, kit*); (3) transducers of growth factor responses (includes GTPases and nonreceptor tyrosine kinases; eg. *ras, raf, src, abl*) and (4) nuclear transcription factors involved in gene expression (eg. *myc, jun, fos*) (reviewed in Hunter, 1991; Cantley *et al.*, 1991). While the functions of all protooncogenes are not fully known, a common theme has arisen which suggests that each is involved in the sequence of events by which growth factors regulate normal cellular growth.

In recent years, particular attention has been paid to the newly-identified receptor tyrosine kinase (RTK)/growth factor receptor family of protooncogenes, which have been

implicated in cell cycle regulation. Characterization of primary structures led to the emergence of a common structural organization for all RTKs, consisting of at least 4 subclasses of structurally-related membrane-spanning polypeptides (reviewed in Yarden & Ullrich, 1988). The three constant features of all RTKs are an extracellular ligand-binding domain in the N-terminal portion of the molecule, a hydrophobic transmembrane domain, and a cytoplasmic domain. It is this latter intracellular portion which confers the tyrosine-specific kinase activity, and exhibits extensive sequence homology both among subclass members and within the entire RTK family (Hunter & Cooper, 1985).

The importance of growth factor receptor-ligand systems has also been considered in the context of oncogenesis. The first evidence for this concept was presented by Waterfield *et al.* (1983), who demonstrated that the oncogene v-*sis*, which encodes for a platelet-derived growth factor-B chain (PDGF-B) homologue, actively transformed fibroblasts by stimulating the tyrosine kinase PDGF receptor in an autocrine fashion. Therefore, the potential role of simultaneously expressed receptors and activating ligands in cell transformation was established, and has since been proposed for a number of the growth factor receptor and/or ligand-encoding protooncogenes.

2.3 Oncogenes in Ovarian Cancer

Aberrant expression or mutations of the genes encoding for tumour suppressors and protooncogenes have been implicated in the pathogenesis of many cancers, including those of the ovary. Table 1 provides a detailed review of cancer-causing genes that have been demonstrated to be aberrantly expressed, amplified or mutated in ovarian cancers. This list includes oncogenes such as *erb*B, HER-2/*neu*, *myc*, *ras*, *fms*, and the p53 tumour suppressor gene. Due to the generative nature of OSE cells, the likelihood of significant mutations occurring which could be passed on to future generations is much greater in OSE than for other types of epithelium. Therefore, it is not surprising that protooncogene and tumour suppressor gene aberrations are being discovered in ovarian cancer with rapid progress.

TABLE 1: Summary of Cancer-Causing Genes Which are Aberrantly Expressed or Mutated in Ovarian Cancer

GENE	CLASS	EVENT	Frequency in Ovarian cancer	Relation to Prognosis/Tumour Characteristics	REFERENCES
c-*erb*-B	RTK oncogene; EGF-R growth factor receptor	overexpressed	≤77%	contradictory: poor/better survival	Berchuck *et al.*, (1990a); Berchuck *et al.*, (1991); Kohler *et al.*, (1989); Bauknecht *et al.*, (1988)
c-*erbB*-2 (neu)	RTK oncogene; HER-2/neu growth factor receptor	amplified/overexpressed	20-32%	poor survival	Slamon *et al.*, (1989); Zhang *et al.*, (1989) Berchuck *et al.*, (1990a); Tyson *et al.*, (1991)
c-*fms*	RTK oncogene; M-CSF-R growth factor receptor	overexpressed	~50%	correlated with CSF-1 levels	Kacinski *et al.*, (1990)
Ki-, H-, and N-*ras*	G protein oncogenes: ras family	overexpressed/amplified mutated	5-10% rare	no correlation	Boltz *et al.*, (1989); Enomoto *et al.*, (1990) Filmus *et al.*, (1986); van't Veer *et al.*, (1988) Rodenburg *et al.*, (1988)
c-*myc*	nuclear transcription factor	amplified	33%	unknown	Baker *et al.*, (1990); Sasano *et al.*, (1990) Serova (1987); Zhou *et al.*, (1988)
BRCA-1	tumour suppressor	mutated	53-77%	unknown	Eccles *et al.*, (1990); Jacobs *et al.*, (1993)
p53	tumour suppressor	overexpression of altered proteins, mutations: exons 5-8	29-80%	detected in all stages; higher % in serous AC and aneuploid cancers	Milner *et al.*, (1993); Mazars *et al.*, (1991) Okamoto *et al.*, (1991)
DOC-2	tumour suppressor? (new)	loss of expression	100%	unknown	Mok *et al.*, 1994, 1998

ABBREVIATIONS:
AC: adenocarcinoma
CSF-1: colony stimulating factor-1
EGF-R: epidermal growth factor-receptor
M-CSF-R: macrophage-colony stimulating factor-receptor
RTK: receptor tyrosine kinase

3. C-*kit* and KL

3.1 Early Discovery

The protooncogene c-*kit* was first identified 12 years ago as the cellular homologue of v-*kit*, the oncogene of the Hardy-Zuckerman 4 strain of feline sarcoma virus (HZ4-FeSV; Besmer *et al.*, 1986; Yarden *et al.*, 1987). HZ4-FeSV was demonstrated to be replication-defective when infected into CCL64 mink cells, but produced distinct foci, grew in soft agar and induced fibrosarcomas when reinfected into nude mice. Thus, the transforming potential of this virus (and, subsequently, its oncogene v-*kit*) was established. V-*kit* shares 58% amino acid homology with v-*fms*, and only weak homology to other oncogenes such as *abl*, *src*, *fgr*, and HER-2/*neu*. (Besmer *et al.*, 1986). Because retroviral oncogenes (v-*oncs*) were thought to represent only segments of c-*onc* mRNAs (c= cellular counterpart), this suggested to the investigators that the 1.1 kb v-*kit* mRNA was missing significant segments of the c-*kit* gene. Examination of the structural features of v-*kit* (in analogy with other transforming viruses) confirmed that HZ4-FeSV was generated by transduction and truncation of the cellular c-*kit* sequence (Yarden *et al.*, 1987). It was not until 1990 however, that the ligand for the Kit tyrosine kinase receptor was identified (Huang *et al.*, 1990; Williams *et al.*, 1990; Zsebo *et al.*, 1990). This pleiotropic growth factor is alternatively known as mast cell growth factor (Williams *et al.*, 1990), stem cell factor (Martin *et al.*, 1990; Zsebo *et al.*, 1990), Kit ligand (KL; Huang *et al.*, 1990) or Steel factor (Witte, 1990), and was identified by virtue of its diverse effects on hematopoiesis, gametogenesis and melanogenesis.

3.2 C-*kit* Structure and Known Functions

Following its discovery, elucidation of the c-*kit* protooncogene structure has revealed that the 5 kilobase (kb) c-*kit* transcript encodes for a 145 kilodalton (kD) protein product which belongs to the subfamily of tyrosine kinase oncogenes that includes the receptors for platelet-derived growth factor (PDGF) and colony stimulating factor-1 (CSF-1) (Majumder *et al.*, 1988; Yarden *et al.*, 1987). Mapped to the long arm of human chromosome 4 (4q31-34; Qiu *et al.*,1988), the nucleotide sequence of c-*kit* predicted a 976 amino acid (aa) polypeptide containing an extracellular N-terminal ligand binding domain, a 23 aa hydrophobic transmembrane stretch, and a 439 residue carboxy-terminal intracellular portion with tyrosine kinase activity (Yarden *et al.*, 1987). Subsequently, several groups showed that c-*kit* was actually the product of the *W* (White-spotting - now known as *Kit*) locus (Chabot *et al.*, 1988; Geissler *et al.*, 1988), mutations of which had been identified much earlier as causing defects in hematopoiesis, gametogenesis, and melanogenesis (Russell *et al.*,1949; Sarvella & Russell, 1956). KL (this author's preference), the stromally-produced growth factor ligand for c-*kit*, was also shown to map genetically to a distinct locus, Steel (*Sl* - now known as *MGF*), which had been shown to be similarly involved in aspects of hematopoiesis, pigmentation, and primordial stem cell migration and growth. In fact, mutations of either the *Sl* or *W* locus produced similar defects in mice, and it was this understanding that allowed investigators to identify KL as the protein product of *Sl* once Kit had been similarly tied to the *W* allele of the mouse.

The loss-of-function mutations of both Kit and KL in *W* and *Sl* mice established this receptor-ligand pair as playing essential roles in proliferation, differentiation, migration, and

survival of Kit-bearing cells. Kit is expressed on many cell types including bone marrow cells, mast cells, germ cells and melanocytes (Lammie *et al.*, 1994). Activation of the Kit receptor by KL stimulates proliferation (Matsui *et al.*, 1991; Metcalf & Nicola, 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 various types of Kit-bearing cells. Additionally, KL-stimulated Kit has been reported to promote mast cell chemotaxis (Meininger *et al.*, 1992), adhesion to extracellular matrix components (Dastych & Metcalfe, 1994; Kinashi & Springer, 1994; Pesce *et al.*, 1997) attachment to fibroblasts (Adachi *et al.*, 1992) and prevention of apoptosis (Iemura *et al.*, 1994; Mekori *et al.*, 1995; Yee *et al.*, 1994).

The kinase activity of Kit protein (p145kit), the c-*kit* gene product, is physiologically dependent upon KL binding, and this receptor-ligand interaction induces receptor dimerization so as to mediate the tyrosine kinase signal (Blume-Jensen *et al.*, 1991; Lev *et al.*, 1992). KL induces Kit to autophosphorylate tyrosine residues, and to associate with, phosphorylate and/or activate a variety of downstream signalling molecules which may ultimately affect a number of cellular activities (Okuda *et al.*, 1992). Molecules that have been shown to associate with, or be phosphorylated by, activated Kit include $p21^{ras}$ (Duronio *et al.*, 1992), $p21^{ras}$GTPase-activating protein and phospholipase C (Herbst *et al.*, 1991, 1992), the p85 subunit of phosphatidylinositol 3'-kinase (Serve *et al.*, 1994), members of the MAP kinase family (Okuda *et al.*, 1992; Paulson *et al.*, 1996; Tsai *et al.*, 1993) and Janus Kinase 2 (JAK2; Weiler *et al.*, 1996). In addition, downstream Kit activity has been shown to be negatively regulated by a pair of protein tyrosine phosphatases, PTP1C (also known as SHP-1) and PTP2C (Feng & Pawson, 1994), and by protein kinase C (PKC) via distinct

mechanisms. Interaction of *src*-homology domains (SH2) on either of the PTP phosphatases with specific tyrosine residues on Kit causes dephosphorylation and hence inactivation of the Kit receptor. PKC inhibits autophosphorylation and kinase activity by phosphorylating activated Kit serine residues, which causes inhibition of KL-induced mitogenic effects (Blume-Jensen *et al.*, 1993). Therefore, PKC, PTP1C and PTP2C are negative regulators of Kit-mediated cellular functions.

The genes for both Kit and KL have been shown to be alternatively spliced into distinct mRNA transcripts, both of which are initially translated into transmembrane products (Huang *et al.*, 1990; Turner *et al.*, 1995). One of these transmembrane products remains membrane-bound, while a soluble form is derived by proteolytic cleavage. During the development of various cell types, differential expression of the two distinct forms of KL provide different mechanisms for controlling and modulating Kit function. For example, the importance of the membrane-bound form of KL for normal ovarian function has been demonstrated by the sterility of Sl^d mice which express only soluble, and not transmembrane forms of KL (Nakayama *et al.*, 1988). The function of the soluble form of Kit is less well understood, although it has been suggested that this form of the receptor may act as an endogenous antagonist to Kit activity by competing with membrane-bound Kit for association with soluble or membrane-bound KL (Turner *et al.*, 1995).

3.3 Evidence for an Oncogenic Role for c-*kit*

C-*kit* is expressed in a number of normal human tissues, including bone marrow cells, mammary ductal cells, neuroglial cells, melanocytes and mast cells. More recently, however,

a variety of cancers have also been shown to express c-*kit*, including glioblastoma, germ cell cancer, and small cell lung cancer. A brief review of some of the cancers in which aberrant c-*kit* (and/or KL) expression have been identified are presented in Table 2. This table includes information on Kit and KL expresssion in various cancers at both the mRNA and protein levels, and corresponding expression (if any) in normal tissues from which the tumours are derived.

TABLE 2: Summary of c-*kit* Expression in Cancers of Various Origins

Cancer (Tissue of Origin)	**Identified in Normal Tissues: Y/N (source)**	**Expression in Tumours**	**Frequency of c-*kit* in cancer***	**References**
lung	N (protein)	mRNA protein	34/77 (12) 14/46	Natali *et al.*, (1992a); Hibi *et al.*, (1991); Hida *et al.*, (1994); Lammie *et al.*, (1994)
thyroid	Y (protein)	protein	2/14	Natali *et al.*, (1992a); Lammie *et al.*, (1994)
brain	Y (protein)	mRNA protein	8/18 (8) 3/36	Cohen et al., (1994); Natali et al., (1992a); Lammie *et al.*, (1994)
melanocytes	Y (both)	protein	22/31	Natali *et al.*, (1992a)
bladder	Y (protein)	protein	3/10	Natali *et al.*, (1992a); Lammie *et al.*, (1994)
cervical	N (protein)	mRNA protein	4/11 (3) 3/20 (3)	Inoue *et al.*, (1994)
breast	Y (protein)	mRNA protein	9/11 (8) 10/80	Hines *et al.*, (1995); Natali *et al.*, (1992a); Lammie *et al.*, (1994)
testis	Y (protein)	mRNA protein	26/32 48/68	Strohmeyer (1994); Natali *et al.*, (1992a); Izquierdo *et al.*, (1995)

LEGEND:
* number in parentheses = number of cases showing coexpression of KL in tumour (not determined for all)
ND = not determined

It has been postulated that protooncogene involvement in cancer may be related to ligand-receptor co-expression, and, furthermore, that ligand binding to surface receptors on tumour cells may enhance cell growth by acting in an autocrine/paracrine fashion. The possibility of autocrine stimulation by the Kit/KL growth factor system in cancer cell growth has been demonstrated for breast cancer (Hines *et al.*, 1995), neuroblastoma (Cohen, *et al.*, 1994), germ cell testicular tumours (Strohmeyer, 1994), small cell lung cancer (Hibi, *et al.*, 1991; Krystal *et al.*, 1996) and cervical cancer (Inoue *et al.*, 1994). While the information regarding the expression and activity of c-*kit* and KL in various cancers is not complete, there is some suggestion that the Kit/KL system may be involved in tumourigenesis, simply based on its relative absence from various normal organs, and its aberrant expression in corresponding tranformed tissues.

Furthermore, an oncogenic role for c-*kit* has been demonstrated following transfection of a c-*kit* construct into NIH 3T3 fibroblasts, testicular Leydig cells and a breast carcinoma cell line, in which transfected cells underwent marked proliferation, transformation and tumour formation (Caruana *et al.*, 1998; Hines *et al.*, 1995; Kondoh *et al.*, 1995; Lev *et al.*, 1990) Therefore, the involvement of the Kit/KL system in ovarian tumourigenesis remains an intriguing possibility.

3.4 Role for *c-kit* in Ovarian Cancer?

In addition to the above cited evidence for a potentially oncogenic role for c-*kit* in cancers of various origin, there have also been some preliminary reports which suggest that c-*kit* may be involved in ovarian tumourigenesis. Varying expression of c-*kit* mRNA and/or

protein in both solid ovarian tumours and ovarian carcinoma cell lines has been documented by a few investigators (Inoue *et al.*, 1994; Natali *et al.*, 1992a; Tonary et al., 1996; A.M. Tonary, personal communication). Co-expression of KL has also been reported for a number of these c-*kit*-expressing tumours (Inoue *et al.*, 1994; A.M. Tonary, personal communication). In contrast, is the lack of expression of c-*kit* (mRNA or protein) in normal human OSE cells, the cellular origin of nearly 90% of all ovarian cancers (Lammie *et al.*, 1994; Natali *et al.*, 1992a). Normal OSE cells do, however, constitutively express KL.

Therefore, the relative absence of c-*kit* from normal OSE cells, its aberrant expression in ovarian tumours, and its ability to induce marked proliferation and transformation following transfection into non-ovarian cells, suggest an oncogenic potential for the Kit/KL system in ovarian tumourigenesis.

3.5 Modulation of c-*kit* and KL Expression

In addition to the possibility of autocrine/paracrine stimulation of the Kit/KL system in various cancers, it has also been demonstrated that regulation of growth by c-*kit* and KL can be modulated by various hormones and growth factors both produced by, and exerting effects on, the ovary. C-*kit* modulation by growth factors has been demonstrated in a number of cell types. For example, transforming growth factor-β (TGF-β)-mediated suppression of growth occurs through inhibition of c-*kit* in murine and human hematopoietic progenitor cells (Dubois *et al.*, 1994, Heinrich *et al.*, 1995), human leukemic myeloblasts (de Vos *et al.*, 1993; Heinrich *et al.*, 1995), and colorectal carcinoma cells (Bellone *et al.*, 1997). Downregulation of c-*kit* mRNA and protein by exogenous TGF-β is associated with maintenance of a quiescent state

in early human CD34high hematopoietic cells (Sansilvestri *et al.*, 1995). In the hematopoietic system, TGF-β -induces downregulation of KL mRNA and protein levels in stromal cells, in conjunction with similar effects on c-*kit* signals (Heinrich *et al.*, 1995). Similarly, the cytokine tumour necrosis factor α (TNFα) downregulated c-*kit* transcripts in normal and leukemic CD34+ cells and normal bone marrow mononuclear cells; the downregulation was associated with inhibition of KL-mediated proliferation in normal CD34positive cells (Khoury *et al.*, 1994). Exposure of human umbilical vein endothelial cells to the inflammation-causing cytokine IL-1 *in vitro*, results in upregulation of KL and also downregulation of c-*kit* expression (Koenig *et al.*, 1994; König *et al.*, 1997). Furthermore, reduction of c-*kit* mRNA expression and surface receptor numbers occurred following addition of erythroid differentiation factor/activin A to murine erythroleukemia (MEL F5-5) cells (Hino *et al.*, 1995).

While there is limited data about hormonal regulation of c-*kit* and KL, a few reports have emerged from various cell systems. In human and rodent ovaries, investigations of the modulation of KL expression in response to gonadotropins have lead to contradictory results. Human chorionic gonadotropin (hCG) and FSH induced significant increases in KL mRNA transcripts in granulosa cells of rats (Ismail *et al.*, 1996) and mice (Motro & Bernstein, 1993) *in vivo*, and cultured primary mouse Sertoli cells were similarly affected by exogenous addition of either FSH or cAMP analogues (Rossi *et al.*, 1993). However, *in vitro* cultures of human granulosa-luteal cells showed inhibition of KL mRNA expression by FSH or hCG in a time- and concentration-dependent manner (Laitinen *et al.*, 1995). In addition, expression of Kit in murine oocytes and theca cells was decreased in response to hCG (Horie

et al., 1991; Motro & Bernstein, 1993). These results indicate that the differing effects of gonadotropins on KL expression may be attributed to differences in species and/or cell type.

Expression of this receptor-ligand pair was also modulated by agents which elevate intracellular cAMP, the downstream second messenger for both gonadotropin receptors. C-*kit* expression was increased in response to protein kinase modulators such as dibutyryl-adenosine-3':5'-monophosphate, cyclic (dbcAMP) or forskolin in malignant hematopoietic cells (Ogawa *et al.*, 1995) and differentiated F9 teratocarcinoma cells (Nishina *et al.*, 1992), and downregulated by the protein kinase C (PKC) activator 12-O-tetradecanoylphorbol-13-acetate (Asano *et al.*, 1993; Nishina *et al.*, 1992). Likewise, dbcAMP induced increased KL mRNA accumulation in *in vitro* systems of mouse granulosa cells (Packer *et al.*, 1994) and Sertoli cells (Tajima *et al.*, 1993).

Therefore, while c-*kit* and KL expression are differentially modulated by a number of factors, and in a number of cell types, there has been no demonstration of regulation of c-*kit* or KL in ovarian cancer cells.

4. Ovarian Surface Epithelial (OSE) and Ovarian Carcinoma Cells

4.1 Establishment of Primary and Immortalized Cells

Due to the lack of appropriate animal models for the study of ovarian cancer, most of the current knowledge regarding OSE cell characteristics, regulation and involvement in ovarian cancer have come from studies using normal and malignant primary OSE cultures and immortalized ovarian carcinoma cell lines. Normal human OSE primary cultures are routinely derived from scrapings of normal human ovaries at the time of surgery for

gynecological reasons, which remove the single-cell layer of surface epithelium and allows the cells to be established as a monolayer in culture (Kruk *et al.*, 1990; Lounis *et al.*, 1994). Ovarian carcinoma cells are usually obtained from pieces of solid tumours (Conover *et al.*, 1998; Lounis *et al.*, 1994; Yuan *et al.*, 1997), but may also be removed from collections of ascites, which are known to contain extruded malignant cells (Hirte *et al.*, 1994; van den Berg-Bakker *et al.*, 1993; Yuan *et al.*, 1997). In 1985, a rat OSE cell line was derived from normal rat tissue (ROSE 199), and this cell line has been used extensively to characterize the derivation of ovarian tumours from OSE (Adams & Auersperg, 1985). According to the most recent literature, culture of normal OSE from human ovaries has been limited to finite primary cultures; however, numerous human ovarian carcinomas have been established as continuous cell lines following spontaneous immortalization. Due to the ready availability, ease of handling, and less sensitive nature of immortalized cell lines in culture, many studies have been carried out using ovarian carcinoma cell lines as *in vitro* models for ovarian cancer. These continuous cell lines are generally easier to manipulate in culture than cells from primary cultures, and due to their longevity, the potential timeframe available for characterization of their regulation by factors which regulate growth and differentiation is considerable, compared to similar analyses in primary cultures. However, as with all immortalized cell lines, ovarian carcinoma cell lines may represent considerable genetic drift from their original tissue source, and therefore characterization of these cells must be extensively related to similar effects in primary cultures, in order to confirm their relevance to ovarian cancer *in vivo*.

4.2 Modulation of Ovarian Carcinoma Cells by Hormones and Growth Factors

4.2.1 Growth Factors

In the time leading up to ovulation, the rapid proliferation of follicular cells contained within the dominant follicle requires the concerted actions and local synthesis of several well-characterized growth factors. These growth factors are present in the follicular fluid of the maturing follicle, and are afforded close contact with the OSE once the follicle has ruptured and the fluid bathes the ovarian surface. Normally quiescent OSE then become regulated by ovarian-produced factors to proliferate and repair the ovulatory wound. This involves the concerted efforts of various stimulatory or inhibitory cytokines and growth factors which may act in autocrine, paracrine or juxtacrine fashion to alter OSE cell growth. These include stimulatory cytokine/growth factors such as interleukin (IL-1), TNF-α, TGF-α and EGF, or the well-characterized growth inhibitor, TGF-β. All of these ovarian factors are produced either by the epithelial cells themselves (EGF, TGF-α/β) or locally by neighbouring follicular or inflammatory cells (IL-1 and TNF-α) (reviewed by Bast, 1993). It is believed that many of these factors may contribute to ovarian malignancies by altering the levels of autocrine/paracrine/juxtacrine control of OSE growth.

The provision of TNF-α for 24 h, either exogenously or via endogenous induction, to four ovarian cancer cell lines triggered immediate amplification of TNF-α and subsequent growth stimulation (Wu *et al.*, 1993). Addition of interleukins (IL-1 and IL-6) to the same four cell lines, as well as additional cell lines, manifested similar effects on cell proliferation, whereas, other factors contained within follicular fluid such as macrophage-colony

stimulating factor, granulocyte-colony stimulating factor, and granulocyte/macrophage-colony stimulating factor, did not alter cancer cell growth (Wu *et al.*, 1992).

Additionally, differential effects have been observed in ovarian carcinoma cells cultured with EGF and TGF-α. EGF is a potent mitogen for many cell types, including those of epithelial origin. TGF-α is a small 50 amino acid protein which structurally and functionally resembles EGF, and can bind to and activate the EGF-R (in some cells, TGF-α is a more potent agonist than EGF) (Lyons & Moses, 1990; Salomon *et al.*, 1990). The majority of ovarian carcinoma cell lines investigated were growth-stimulated in the presence of either of these two growth factors (Berchuck *et al.*, 1990b; Jindal *et al.*, 1994; Khan *et al.*, 1997; Stromberg *et al.*, 1992; Wimalasena *et al.*, 1992). Indeed inhibition of cell growth occurred in the presence of a TGF-α-neutralizing monoclonal antibody (Stromberg *et al.*, 1992), and this effect may have been associated with significant reductions in the protooncogene HER-2 protein levels (Marth *et al.*, 1992). However, a single report also indicates that inhibition of ovarian carcinoma cell proliferation and DNA synthesis may also occur upon addition of TGF-α or EGF (Zhou & Leung, 1992).

TGF-β has been demonstrated to inhibit the proliferation of various primary cultures of both normal OSE and epithelial ovarian cancer cells obtained from ascites (Berchuck *et al.*, 1992; Havrilesky *et al.*, 1995; Hurteau *et al.*, 1994), and a small subset of ovarian carcinoma cell lines (Berchuck *et al.*, 1990b; Kim *et al.*, 1997; Yasui *et al.*, 1997). This is consistent with its role as a powerful growth inhibitor in many cell types, including epithelial cells (Lyons & Moses, 1990). Inhibition of ovarian carcinoma cell line growth by TGF-β was also shown to result in rapid increases in the expression of genes for c-*fos* and c-*jun*, with concurrent

down-regulation of those for c-*myc* and the anti-apoptotic gene *bcl*-2 (Kim *et al.*, 1997; Lafon *et al.*, 1996).

The presence of receptors for EGF/TGF-α and TGF-β (Crew *et al.*, 1992; Gordon *et al.*, 1995; Jakowlew *et al.*, 1997; Jindal *et al.*, 1995; Zhou & Leung, 1992) in a number of ovarian cancer cell lines, and the concomitant secretion of these growth factors (Berchuck *et al.*, 1990b) by a number of ovarian cancer cell lines have further substantiated a potential role of growth factors in modulating ovarian carcinoma growth (Crew *et al.*, 1992; Jakowlew *et al.*, 1997; Jindal *et al.*, 1995; Zhou & Leung, 1992).

4.2.2 Ovarian Steroids and Gonadotropins

In the developing fetal ovary, marked OSE proliferation at 16 to 20 weeks coincides with the appearance of steroid-producing cells in the ovarian cortex (Gondos, 1975). Since the ovary is both a steroid-producing and a steroid-responsive organ, the effects of hormones on malignant OSE cells is of interest.

Receptors for estrogen, progesterone, and testosterone have been identified with varying levels of expression and activity in many ovarian cancer tumours (Kobayashi *et al.*, 1996; Kommoss *et al.*, 1992) and human ovarian carcinoma cell lines (Grenman *et al.*, 1994; Hamilton *et al.*, 1983; Kobayashi *et al.*, 1996; Kuhnel *et al.*, 1987). In some reports, estrogen receptors were preferentially localized to more highly differentiated malignant tumours (Iversen et al., 1986). In others, metastatic tumours contained fewer receptors than the original primary tumour (Quinn *et al.*, 1988), while other investigators found no significant differences in estrogen or progesterone receptor numbers amongst the four clinical stages

(Vihko *et al.*, 1983).

The effects of steroids on primary tumours and ovarian cancer cell lines have also been investigated. Administration of estradiol (E_2) predominantly causes stimulation of growth in a number of ovarian carcinoma cell lines (Langdon *et al.*, 1990; 1993; 1994; Nash *et al.*, 1989; Pavlik *et al.*, 1991; Simpson *et al.*, 1998; Wimalasena *et al.*, 1993); however, suppression of growth in response to E_2 has been demonstrated in a primary culture of an epithelial ovarian cancer (Wimalasena *et al.*, 1992), indicating that the effects of E_2 on ovarian cancer cell proliferation are not well defined.

Limited data is available on the effects of testosterone and progesterone on ovarian carcinomas *in vitro*. Conflicting reports have indicated that the addition of progesterone (P_4) to primary tumour cultures stimulated the release of EGF/TGF-α and was associated with a tendency for growth stimulation (Ridderheim *et al.*, 1994), while addition of P_4 to 3AO and AO ovarian carcinoma cells was associated with anti-proliferative effects, including marked apoptosis and upregulation of the p53 tumour suppressor gene (Bu *et al.*, 1997). Interestingly, testosterone stimulated the growth of OSE cells in guinea pigs, and caused the formation of benign cysts, adenomas and papillomas within and on the surface of the ovary (Silva *et al.*, 1997).

The gonadotropic hormones, follicle stimulating hormone (FSH) and luteinizing hormone (LH), are glycoproteins which are released from the anterior pituitary, and exert their effects via binding to specific membrane-bound gonadotropin receptors (FSH-R and LH-R, respectively) on ovarian cells. Bound gonadotropin receptors (GR) interact with stimulatory G proteins (G_s) which activate adenylate cyclase to promote cAMP formation. These

hormones are cyclically elevated each month at the time of ovulation, for some investigators have suggested the "excessive gonadotropin" theory, which states that the trophic effects of gonadotropins on OSE cells played a prominent role in the generation of OSE malignancy. This hypothesis is directly tied to another school of thought, namely that some aspect of menopause, and most likely, the marked changes in hormonal levels (particularly those of the significantly elevated gonadotropins) during that time, are responsible for the transformation of OSE cells. Numerous studies have provided support for this theory, specifically indicating the predominantly stimulatory effects of both gonadotropins on OSE cells and ovarian cancer cells, as described below.

Gonadotropin receptors have been identified on benign and malignant ovarian tumour cells (Kammerman *et al.*, 1981; Kobayashi *et al.*, 1996; Mandai *et al.*, 1997) and on ovarian cancer cell lines (Mandai *et al.*, 1997). Enhanced cell proliferation in response to LH/hCG and/or FSH occurred in primary rabbit OSE cells (Osterholzer *et al.*, 1985), primary human ovarian carcinoma cell cultures (Kurbacher *et al.*, 1995; Wimalasena *et al.*, 1992) and ovarian carcinoma cell lines (Feng *et al.*, 1996; Simon *et al.*, 1983; Wimalasena *et al.*, 1993). In another report, hCG did not increase cell proliferation in 2 ovarian cancer cell lines examined; however, addition of hCG significantly suppressed cisplatin-induced apoptosis in one of the cell lines (Kuroda *et al.*, 1998). Therefore, varied studies have demonstrated a strong role for gonadotropins in supporting, and perhaps initiating ovarian tumourigenesis.

4.2.3 cAMP Pathway Stimulators: Dibutyryl cyclic AMP, 8-bromo-cAMP and Cholera Toxin

Second messengers act to regulate target functions specifically and, thereby, transduce extracellular events into functional cellular activities. The target elements of second messengers include various molecules involved in diverse cellular activities such as protein kinases, phosphatases, proteases and ion channels. Cyclic AMP was the first of the class of small molecules acting as second messengers to be understood (Rall *et al.*, 1957). Cyclic AMP is involved in a cascade of events which begins following receptor activation by ligand binding, and culminates in the occurrence of specific cellular events. The process begins when G-protein coupled receptors (such as gonadotropin receptors) activate key effector molecules which transduce the action of extracellular ligands. In this case, G-proteins regulate a key effector enzyme known as adenylate cyclase, which in turn catalyzes the formation of cAMP from ATP. Resting cAMP levels are usually very low, and activation of adenylate cyclase results in abrupt, albeit transient, elevations in intracellular cAMP levels. During this brief period of increased signal generation, cAMP activates the catalytic subunit of protein kinase A (cAMP dependent protein kinase A), and then is rapidly metabolized by cAMP phosphodiesterase. Protein kinase A functions by phosphorylating proteins on serine and threonine residues, and has been implicated in a number of cellular processes such as mitogenesis, cellular differentiation, hormone release, and neurotransmission (Hannun & Bell, 1993).

DbcAMP and 8-bromo-adenosine-3':5'-monophosphate, cyclic (8-bromo-cAMP) are membrane-permeable agonistic analogues of cAMP. Once they have traversed the membrane,

these two molecules artificially mimic adenylate cyclase-induced cAMP formation by virtue of their cAMP moieties. Cyclic AMP provided by these two compounds is identical in structure and function to the endogenous signalling molecule. Cholera toxin (CT) is an adenylate cyclase activator. Following binding to a plasma membrane receptor (ganglioside GM1), CT is internalized and subsequently activates adenylate cyclase, and results in *de novo* cAMP formation.

While the effects of cAMP and its analogues on normal OSE cells and ovarian cancer cells is of interest, limited studies have examined their role in modulating the proliferation of these cell types. One report, obtained from a cultured ovarian carcinoma cell line, demonstrated the ability of dbcAMP to block DNA synthesis in cells that were pre-treated with steroidogenesis-inducing protein (Khan *et al.*, 1997).

In addition, several investigators have demonstrated the effects of cAMP on other tumours (not ovarian cancer). Addition of dbcAMP and/or adenylate cyclase activators such as forskolin or CT caused dose-dependent decreased proliferation in TE85 human osteosarcoma cells (Mohan & Baylink, 1991), human meningiomas (Huttner *et al.*, 1996), A431 epidermoid carcinoma cells (Kamiya *et al.*, 1995), histiocytic lymphoma cells (Laskin *et al.*, 1990) and human pancreatic cancer cells (Ohmura *et al.*, 1993).

5. HEY and OVCA 429 Ovarian Carcinoma Cell Lines

The purpose of this thesis was to study the regulation of proliferation and c-*kit* and KL expression in the HEY and OVCA 429 ovarian carcinoma cell lines. The HEY cell line was originally derived from a murine xenograft of a disaggregated human ovarian tumour, grown from a peritoneal deposit of a moderately differentiated papillary cystadenocarcinoma of the ovary (Buick *et al.*, 1985). This monolayer cell line was established after growing xenograft HX-62 (passage 3; Selby *et al.*, 1980) in immunologically deprived CBA/CJ mice. HEY cells were found to exhibit the characteristic morphology of epithelial ovarian tumours under light and electron microscopy, and demonstrated resistance to cis-platinum, a chemotherapeutic agent commonly used in ovarian cancer patients (Buick *et al.*, 1985). HEY cells secrete (and are stimulated by) TGFα, possess TGFβ type II, betaglycan, and endoglin receptors (types of TGF-β receptors), and bind TGFβ1 (Jindal *et al.*, 1994; 1995). Growth stimulation of HEY cells by granulocyte-colony stimulating factor is accompanied by increases in transcript expression of genes involved in mitogen signalling (JNK1 and 2 and ERK1 and 2) and early gene expression (nuclear transcription factor ; c-*jun*) (Brandstetter *et al.*, 1998; Spinner *et al.*, 1995). Ascitic fluid from ovarian cancer patients containing an unidentified growth factor has been shown to induce proliferation of HEY cells, as assessed by [^{3}H]-thymidine incorporation and colony formation in methylcellulose (Mills *et al.*, 1988). Furthermore, HEY cell tumours can be established intraperitoneally in immunodeficient nude mice, in the presence of ascitic fluid (Mills *et al.*, 1990).

Compared to the amount of information available for HEY cells, considerably less characterization of the OVCA 429 ovarian carcinoma cell line has been reported. However,

available studies have revealed that these cells express mRNA for the EGF-R (Gordon *et al.*, 1995), and produce, but are unaffected by, TGF-β (Berchuck *et al.*,1992b). As well, OVCA 429 cells have normal p53 genes, and are resistant to the apoptosis-inducing effects of the ovarian chemotherapeutic agents cisplatin, cyclophosphamide and paclitaxel (Havrilesky *et al.*, 1995).

Both HEY and OVCA 429 cells express detectable levels of c-*kit* and KL mRNA, as determined by RT-PCR and Northern analysis (A.M. Tonary, personal communication), and therefore these ovarian carcinoma cells provide an ideal system in which to study the regulation of c-*kit* and KL by ovarian factors.

6. Rationale

The purpose of this thesis was to explore the regulation of c-*kit* and KL expression in ovarian carcinoma cells by a variety of factors which are produced by, and/or act on the ovary, in order to determine the role of this growth factor receptor/ligand system in ovarian cancer progression. While c-*kit* is *not* expressed in normal OSE, the majority of ovarian cancers do express c-*kit*, and many of these also maintain co-expression of the ligand. It is well substantiated that the incidence of ovarian cancer rises significantly during menopause, when any number of changes in the ovarian milieu (such as hormones) could conceivably influence OSE transformation. Furthermore, although very little is known about the regulation of c-*kit* in OSE cells, a number of hormones, growth factors and cytokines have been shown to regulate the expression of c-*kit* in other proliferating cells. Since autocrine/paracrine signalling is a demonstrated means of transformation and uncontrolled cell growth, and an

oncogenic role has been suggested for c-*kit* in other cancers, it is hypothesized that the Kit/KL system may provide a means for proliferation and/or transformation of OSE cells, under the regulation of ovarian factors.

For these studies, two continuous ovarian carcinoma cell lines (HEY and OVCA 429) provided an *in vitro* system to model ovarian cancer *in vivo*, in order to study the regulation of transformed OSE cells. The HEY and OVCA 429 cell lines have been demonstrated to express both c-*kit* and KL mRNA, and therefore, the coexpression of this growth factor receptor-ligand pair provided an ideal opportunity for potential manipulation of the Kit/KL system in ovarian cancer cells, for determination of its involvement in ovarian cancer. Because immortalized cell lines are not always the most clinically relevant *in vitro* models for tissues *in vivo*, an attempt was made to establish human primary ovarian tumour cells in culture, in order to compare the effects of growth factors and hormones on both cell systems. The specific objectives of this study were the following:

1. To determine the effects on HEY and/or OVCA 429 cell proliferation of the following: growth factors (EGF, TGF α and TGF-β), steroids (E_2, P_4 and T) and gonadotropic hormones (FSH, hCG), and agents which elevate cellular cAMP (dbcAMP, 8-bromo-cAMP, and cholera toxin).
2. To determine the parallel effects of the aforementioned factors (which are produced by, or exert effects on, the ovary) on c-*kit* and KL expression in HEY and OVCA 429 cells.
3. To determine whether any factor-induced alterations in ovarian cancer cell proliferation are linked to changes in the expression of c-*kit* and/or KL.

4. To establish primary cultures of human ovarian carcinoma cells, as a means of providing a Kit-positive *in vitro* cell system which is immediately descended from the original tumour source. This would allow the relative responses of primary ovarian carcinoma cells to be compared to those of the continuous cell lines, in terms of the effects by the aforementioned growth factors, hormones or agents which elevate cAMP, on cell proliferation and c-*kit*/KL expression.

MATERIALS AND METHODS

7. Tissue Culture

7.1 Maintenance of Ovarian Carcinoma Cell Lines in Culture

Two ovarian epithelial cancer cell lines, HEY and OVCA 429, were obtained from Dr. Gordon Mills (Houston, Texas). The human small cell lung cancer line, ACC-LC-80 (LC-80), which expresses abundant c-*kit* mRNA was used as a positive control for the expression of c-*kit* mRNA (Hibi *et al.*, 1991), whereas LC-73 cells (KL positive/c-*kit* negative) were used as a negative control for c-*kit* expression, and positive for KL mRNA (A. Tonary, unpublished findings). Both lung cancer cell lines were generously provided by Dr. Douglas Gray (Ottawa, Canada). OV2008 ovarian carcinoma cells, which do not express either transcript (A.M. Tonary, personal communication) were used as a true negative control for both c-*kit* and KL expression and were also obtained from Dr. Mills. For maintenance and experiments, all cells were maintained in 100 mm tissue culture dishes (100 x 20 mm Nalge Nunc International, Denmark) in culture medium consisting of α- minimal essential medium with phenol red dye (α-MEM; Gibco BRL, Grand Island, N.Y.), plus 10% heat-inactivated fetal calf serum (FCS; CanSera, Rexdale, ON), while the OVCA 429 cells received additional supplementation with 1% non-essential amino acids solution (NEAA; Gibco BRL). All cell lines were routinely subcultured every 2-4 days upon reaching confluence. Cells were washed in phosphate-buffered saline (PBS), collected with 0.025% trypsin/1 mM ethylenediaminetetraacetic acid (EDTA) in PBS, and centrifuged (3000 rpm for 3-5 minutes) following trypsin-EDTA inactivation. Pelleted cells were resuspended in fresh media and cell number was determined by calculating the mean of two independent counts using a

hemocytometer. The cells were cultured at 37°C in an incubator equilibrated with 5% CO_2/95% O_2.

Periodically, cells were prepared for long-term storage by resuspending pelleted cells in a freezing medium consisting of base medium supplemented with 10% dimethyl sulfoxide (DMSO; Sigma Chemical Co., St. Louis, MO). Cells were aliquoted (0.5-2.0 x 10^6 cells/ml) into specialized cryogenic vials (Nalge Co., Rochester, N.Y.) and transferred on dry ice to a -80°C freezer for short-term storage (days to weeks) so as to minimize the damaging effects of instantaneous freezing. Frozen aliquots were eventually transferred to liquid nitrogen for storage longer than a few weeks. To thaw cells, frozen vials were transferred directly from storage and gently agitated in a 37°C waterbath until thawed. The contents were then transferred to a 15 ml Falcon tube and diluted 1:10 with serum-containing medium. Diluted cells were centrifuged (3000 rpm for 3-5 min), resuspended in fresh culture medium and transferred to a 60 mm tissue culture dish (60 x 15 mm; Nalge Nunc International).

7.2 Hemocytometry

Quantitative assessments of cell number for all cultures were completed using hemocytometers (Improved Neubauer). Prior to each use, hemocytometers were washed with tap water, rinsed with 70% ethanol and dried using lint-free tissues (KimWipe). The mean number of cells was determined by calculating the average of two independent hemocytometer counts for each dish. Briefly, 10 µL cell suspension aliquots were transferred to the edge of a hemocytometer coverslip and allowed to be drawn across the grid surface by capillary action. Using a 10X objective, all cells within the central 1 mm^2 grid were counted, and the

resultant value was multiplied by 10^4 and by the resuspension volume, *e.g.*;

$$50 \text{ cells} \times 10^4 \times 1 \text{ ml} = 50 \times 10^4 \text{ cells (total)}.$$

If fewer than 20 cells were counted in the central 1 mm^2 grid, then a recount was completed which included the four larger corner squares in addition to the central one, and the final cell count used was taken as an average of these five squares.

7.3 Trypan Blue Viability Test

The trypan blue dye exclusion test was used to measure viability of cells cultured in some of the treatments. This viability test relies on the principle that viable cells are normally impermeable to trypan blue (Kaltenbach *et al.*, 1958), but that a breakdown in membrane integrity, such as that observed in dead or dying cells, will enable dye uptake and therefore allow for the easy distinction between viable and non-viable cells. This test was used only as a gross indicator of cell viability among different treatment groups, and was incorporated into the hemocytometry protocol. Prior to counting, trypan blue dye (0.4% stock; Gibco BRL) was added at a final concentration of 1:14 to thoroughly resuspended cells, and 10μL aliquots were processed as described above (section **7.2**). Percent viability was then determined by calculating the ratio of unstained cells/total number of cells counted.

8. Experimental Protocols

8.1 General Experimental Design

To study the effects of hormones, cAMP and growth factors on ovarian cancer cells, all experiments followed a general experimental protocol. HEY or OVCA 429 cells were grown to subconfluence (65-85%) in culture medium (α-MEM + 10% FCS ± 1% NEAA), collected with trypsin-EDTA and total cell number was determined using a hemocytometer. HEY cells were initially plated at a density of 5×10^4 cells in 60 mm dishes (1×10^4 cells/ml), and OVCA 429 cells, a slower growing cell line, were plated at 7.5×10^4 cells/60 mm dish (1.25×10^4 cells/ml). Cells were incubated at 37°C for 2 hours to allow for cell attachment prior to experimentation. Control dishes received an equivalent volume of culture medium, and were handled in the same manner as treated dishes. All dishes were coded to allow for blinded assessments. After the 48 h culture period, all groups were assessed for confluency and morphological changes, using an Olympus CKZ inverted microscope (Carsen Group, Markham ON) under 4,10 and 20X objectives. Cells were subsequently collected in trypsin-EDTA, centrifuged at 3000 rpm for 5 min and thoroughly resuspended in 1 ml of media. Final number of viable cells for each dish was determined by hemocytometry.

All treatment groups were plated in triplicate and each experiment was completed at least three times. Cells within each treatment group were subsequently pooled, pelleted at 3000 rpm (5 min), and prepared for RNA or protein extraction as indicated (see sections **9.1** and **10.1** for details). Briefly, pooled samples were washed with PBS, centrifuged once again, and then cell pellets were either frozen in liquid nitrogen for storage at -80°C, or they were lysed directly into RNA lysis buffer (section **9.1**). Ultimately, either RNA or protein was

collected from all of the pooled samples, and subjected to Northern or Western analysis respectively. Northern blotting was performed to quantitate the amount of c-*kit* and KL mRNA expressed by HEY and OVCA 429 cells in response to the various treatments. Any variations to this general protocol are detailed within the subsequent sections.

8.2 Specific Experimental Protocols

8.2.1 Effects of Growth Factors

Studies to assess the effects of EGF, TGF-α and TGF-β on HEY cell proliferation and gene expression were carried out as described below (sections **8.2.1 A -B**). Variations to the general experimental design (section **8.1**) included: differences in media FCS concentrations and initial seeding densities, and the inclusion of a 24 h time period for some of the cultures.

(A) 10% FCS-Containing Cultures

HEY cells were plated at an initial seeding density of 5×10^4 cells in 60 mm dishes and cultured in the presence or absence of 10 ng/ml EGF (epidermal growth factor; Boehringer Mannheim), 10-20 ng/ml transforming growth factor-alpha (TGF-α; R&D Systems, Minneapolis, MN), or 10 ng/ml transforming growth factor-$beta_1$ (TGF-β; R&D Systems, Minneapolis, MN) for a 48 h period. Stock solutions of EGF (5 μg/ml; derived from mouse submaxillary gland) were made up in sterile PBS containing 1 mg/ml bovine serum albumin (BSA) and stored in 20 μL aliquots at -80°C. Recombinant human TGF-α was prepared in 10 mM sterile acetic acid with 0.1% BSA to achieve a working stock solution of 10 ng/μL which was kept at -20°C for several months. TGF-β (recombinant, human) was

reconstituted in 4 mM sterile HCl, 0.1 % BSA to yield a 20 ng/μL stock (stored at -20^{0}C storage), which was further diluted 40-fold in culture media to minimize any potentially toxic effects associated with HCl (preliminary studies with cells cultured in media containing similarly diluted 4 mM HCl in the absence of growth factor ensured the absence of toxicity). All three growth factors, at a final concentration of 10 ng/ml, were added to culture medium containing 10% FCS. Following determination of final cell numbers, all samples were pooled and RNA extracted as described in section **9.1.**

(B) 1% FCS-Containing and FCS-Free Cultures

Additional studies were performed to eliminate the possibility that FCS was masking the effects of exogenous growth factors on proliferation. These experiments involved reducing the FCS concentration of the culture medium. In 1% serum-containing cultures, HEY cells were cultured in the presence of growth factors (as described above), except that the initial seeding density was increased to 7.5 x 10^4 cells/60 mm dish to compensate for the diminished growth of these cells in reduced concentrations of FCS. Cells were counted after 24 or 48 h of culture. Further studies were carried out using FCS-free media, in which HEY cells were cultured in the presence or absence of EGF, TGF-α or TGF-β for 24-48 h in culture medium consisting of α-MEM only. Due to the poor viability of HEY cells in FCS-free conditions, additional modifications to the medium were introduced. These included the addition of: 4 mM glutamine, 1 mg/ml BSA, or insulin-transferrin-selenium supplement (ITS; 5 μg/ml insulin and transferrin, 5 ng/ml selenium), either alone, or in combination.

8.2.2 Effects of Gonadotropins and Steroid Hormones

To examine the hormonal regulation of proliferation and gene expression in ovarian carcinoma cells, HEY and OVCA 429 cells were cultured for 48 h in the presence or absence of 120 ng/ml FSH (NIADDK-oFSH-19), 5 IU/ml hCG (Sigma Chemical Co., St. Louis, MO) or 500 nM estradiol-17β (E_2), progesterone (P_4) or testosterone (T) (all from Sigma). All three steroid solutions were made up as 0.7-0.9 mg/ml stocks in ethanol which were diluted in culture media to yield the final 500 nM concentration. Lyophilized FSH was reconstituted in Dulbecco's phosphate buffered saline (D-PBS) + 0.5% bovine serum albumin (BSA), and lyophilized hCG from pregnant human urine was reconstituted in sterile water (500 IU/ml) and added directly to the cultures. Human CG is structurally and functionally similar to LH, binds to the LH-R with equal or greater affinity than does LH, has a longer half-life and is less expensive and more readily available than LH. Samples from all six treatment groups (FSH, hCG, E_2, P_4, or T-treated, and control cells) were prepared for RNA extraction and analysed by Northern blotting techniques.

For some of the hormonal experiments, FCS added to the culture media was first depleted of endogenous steroids using charcoal extraction (Vanderhyden, 1988). Briefly, 50 mg/ml charcoal was added to FCS, and the FCS/charcoal solution was stirred overnight to remove all low molecular weight substances including steroids. The charcoal was removed the next day by centrifugation at 1800 x g for 30 min. The resulting supernatant was ultracentrifuged for 60 min at 27,000 x g, and then sterilized by positive pressure filtration (pore size = 0.2 μM) and stored at 4^0C until use. Charcoal-treated FCS was added to media in a 1:1 exchange for regular FCS (i.e. 10% FCS = 10% charcoal-stripped FCS).

8.2.3 Activation of cAMP Signal Transduction Pathway

(A) Dose-Response: DbcAMP and 8-bromo-cAMP

The binding of gonadotropins to their respective surface receptors activates adenylate cyclase which elevates intracellular cAMP, causing activation of cAMP-dependent protein kinases and ultimately promoting gene activation. Studies which were carried out to assess the effects of cAMP analogues on HEY cell proliferation and gene expression were conducted using dbcAMP and 8-bromo-cAMP. These two membrane-permeable molecules, which structurally resemble cAMP, were chosen to experimentally elevate cAMP levels and thereby mimic the activation of the two gonadotropin receptors, FSH-R and LH-R. Plated HEY cells (5×10^4/60 mm dish- see section **9.1**) were cultured for 48 h with either dbcAMP (Boehringer Mannheim, Germany), or 8-bromo-cAMP (Boehringer Mannheim) at the following concentrations: 0, 0.25, 0.5, 1 and 2 mM. Stock solutions of dbcAMP and 8-bromo-cAMP were made in FCS-free media, filter sterilized through a syringe filter (0.2 micron; Nalgene) and supplemented with 10% FCS to yield 40 mM. Volumes which when added to the culture medium would represent the final concentrations were then removed from these 40 mM stock solutions which were stored at 4^0C for up to two months. Pooled samples from these experiments were used primarily for analysis of c-*kit* and KL mRNA expression; however, protein lysates were extracted and analysed by Western blotting procedures in some samples.

(B) Dose-Response: Cholera Toxin

The effects of cholera toxin on HEY cell proliferation and gene expression were also examined under similar conditions as were used for the dbcAMP dose-response studies (section **8.2.3.A**) Cholera toxin (Sigma) was prepared as a 10 mg/ml stock in sterile water and was diluted directly into the culture medium to yield the following final concentrations: 0, 0.001, 0.01, 0.1 and 1.0 μg/ml cholera toxin. The cells were cultured for 48 h prior to cell harvesting, counting and extraction of total RNA for gene expression analysis.

8.2.4 Role of Kit in HEY cell growth following cAMP stimulation

In order to assess the role of Kit expression in cAMP-mediated regulation of HEY cell growth, an anti-Kit monoclonal antibody (SR-1; provided by Larry Bennett, Amgen Inc, Thousand Oaks, CA) was used to block KL-mediated Kit activation. Briefly, 1.25×10^4 cells were seeded into 24-well dishes in 0.5 ml total volume and cultured in the presence or absence of 2 mM dbcAMP (as prepared in **8.2.3.A**), while the remaining wells received media control. Additionally, all of the wells were cultured either with media control, or in the presence of SR-1 at the following concentrations: 1, 2, and 5 μg/ml. SR-1 was prepared as a 0.17 μg/μL stock in sterile water and added directly to the wells. After 48 h, cells were harvested and counted as described (section **8.1**), and viability was assessed using the trypan blue test (**7.3**). All treatment groups were completed in duplicate, and mean cell numbers were derived from two independent experiments.

9. RNA Analysis

9.1 Extraction and Quantification of RNA

Total RNA was extracted from all cell pellets using the Qiagen RNeasy Mini-Kit (Qiagen Inc., Santa Clarita, CA). Basically, 350μL of Qiagen Lysis Buffer RLT with added β-mercaptoethanol (OmniPur, Darmstadt, Germany) was added to cell pellets either immediately following collection from each experiment, or to frozen samples that were thawed at 37^{0}C for 10 min prior to lysis in Buffer RLT. Contaminating DNA was homogenized using 20 gauge (0.9 mm) needles fitted to 1 ml syringes and the resultant samples were processed through alternating wash/spin cycles in specialized binding columns (provided with kit). The final RNA products were eluted in 100 μL of RNase-free water and transferred to eppendorf tubes. RNA was also extracted from human LC-80 (Kit+), LC-73 (KL+) or OV2008 (Kit-/KL-) ovarian carcinoma cells to use as positive and negative controls. RNA concentration was determined by quantifying 1 μL of total RNA in 49 μL (1:50) of RNase-free water (diethylpyrocarbonate; DEPC-treated) using a spectrophotometer (Beckman DU 640 Spectrophotometer). Fifty μL of DEPC-treated water was used as a blank. Using this sample setup for the spectrophotometer, an optical density reading of 1.0 at λ=260 nm would indicate a concentration of ~40μg/ml of total RNA in the sample.

9.2 Northern Analysis

9.2.1 Gel Electrophoresis of RNA Samples

Northern blotting was performed to compare the relative amounts of c-*kit* and KL mRNA expressed by HEY and OVCA 429 cells under different treatment conditions. RNA

samples were prepared for gel electrophoresis by placing 5-15 μg RNA in an eppendorf tube and precipitating with 1 ml of cold ethanol-2% potassium acetate (KOAC) followed by centrifugation (14,000 rpm at 4^0C) for 20 min. The ethanol was aspirated, and the RNA pellets were resuspended in 20-30 μL of sample buffer (50% weight by volume (w/v) formamide, 22.5% formaldehyde, 10% 10X RNA gel buffer and 17.5% DEPC water). Prior to gel-loading, samples were heated at 65^0C for 10 min and cooled on ice for several minutes. Loading sample buffer (DNA loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol) + 10% ethidium bromide) was added 1/10 to samples once they were adequately cooled.

Denaturing formaldehyde-containing gels were prepared by adding 10% w/v 10X gel buffer and 5% w/v formaldehyde to a slightly cooled 0.9% agarose LE (Boehringer Mannheim Canada, Laval, Quebec) solution made in purified water. The resultant gel preparation was poured into a 250ml large gel box and allowed to harden. Upon solidification, 2 L of gel buffer (10% w/v 10X RNA gel buffer (200 mM MOPS, 50 mM sodium acetate·$3H_2O$, 10 mM EDTA), 85% w/v distilled H_2O (dH_2O)) was poured into the gel box (adequate coverage of approximately 0.5-1.0 cm), the lane comb was removed, and the samples were loaded into the gel. Due to the large size of the fragments of interest (5-6 kb), these gels were generally run overnight at 50-60 Volts with a water pump to circulate the buffer during this extended period.

9.2.2 Northern Transfer and Hybridization

Following the overnight run, gels were removed from the apparatus, and the presence

of the RNA was determined by visualization of ethidium bromide-stained RNA under ultraviolet (UV) illumination. A permanent record of these signals was made using a thermal printer (UVP Video Copy Processor; DiaMed, Mississauga, ON), and was used as a preliminary estimate of equal sample loading. RNA was transferred by capillary action overnight in 2X saline sodium citrate (SSC) to a nylon membrane filter (Hybond N; Amersham, Arlington Heights, IL). Following transfer, membranes were crosslinked at 100J by UV irradiation and placed into a glass hybridization tube. Membranes were prehybridized (2- 48h) and hybridized (overnight) at 42^{0}C in a solution containing 50% w/v formamide, 5X Denhardt's, 5X SSPE, 1% SDS and 250 μg/ml denatured herring sperm carrier DNA. Hybridized blots were washed twice in 2X SSC, 0.1% SDS at room temperature (RT) for 15 min, followed by higher stringency washes in 0.2-0.5X SSC, 0.1% SDS (2 x 15 min) at 65^0 prior to phosphorimager cassette exposure.

The human cDNA probes used in these analyses were: i) a 5.1 Kb SalI/NotI fragment isolated from a pSportI plasmid containing a full-length human c-*kit* cDNA, and ii) a 920 bp HindIII/XbaI human KL cDNA fragment isolated from a pGEM3 plasmid, both generously provided by Dr. Frederick Jacobson (Amgen Inc.). Each DNA fragment was separated from the source plasmid by agarose gel electrophoresis (0.9% agarose gel), and purified using GeneClean (BIO 101, La Jolla CA). An α-tubulin cDNA (provided by Dr. M.W. McBurney, Ottawa) was used as an internal loading control. For each probe, 50 ng of cDNA was labelled to >10^9cpm/μg with α-^{32}P-deoxy-CTP (50 μCi) using a random primed DNA labelling kit (Boehringer Mannheim, Germany), precipitated with trichloroacetic acid (TCA), and purified of free nucleotides by repeat centrifugations through Sephadex G-50 fine

spin columns. Probes were denatured for 5-10 min (100^{0}C) and cooled on ice prior to addition to the membranes.

9.2.3 Densitometry

Hybridization signals on the membranes were analyzed after various exposure times using a Molecular Dynamics Phosphorimager with ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Blots were stripped before reprobing in a boiling 0.1% w/v SDS solution and re-exposed to a phosphorimager cassette for an exposure period equivalent to that which they had been previously exposed. Signals were quantitated by calculating the 'signal'/α-tubulin ratios and standardizing them to control values arbitrarily set to 100%.

10. Protein Analysis

10.1 Protein Extraction

Protein lysates were extracted from harvested cells by one of two methods. Most commonly, frozen cell pellets were dispersed in a 1:1 ratio of ice-cold suspension buffer (0.1 M NaCl, 0.01 M Tris·Cl pH 7.6, 0.001 M EDTA pH 8.0, 1 μg/ml aprotinin and 100 μg/ml phenylmethylsulfonyl fluoride) and 2X SDS gel-loading buffer (50 mM Tris·Cl pH 6.8, 200 mM dithiothreitol, 4% electrophoresis grade SDS, (0.2 % bromophenol blue and 20 % glycerol)]. Alternatively, adherent cells were lysed directly in the individual culture dishes by washing cells twice with cold PBS, aspirating any residual wash and adding an appropriate volume (determined by dish size) of hot (85^{0}C) 1X SDS gel-loading buffer (50 mM Tris·Cl pH 6.8, 100 mM dithiothreitol, 2% electrophoresis grade SDS, (0.1% bromophenol blue and

10 % glycerol). The viscous lysate was then scraped into a microfuge tube with a rubber policeman. Following lysis, all samples were heated in a boiling water bath for 10 min, passaged 10 times through a 23 gauge needle to shear the chromosomal DNA, and pelleted at 10,000 g for 10 min at room RT. Supernatants were transferred to a fresh tube, and quantitated by the appropriate assay (see below). During use, protein lysates were kept on ice at all times, and stored at -80^{0}C.

Protein concentration of lysates was quantitated using commercially available protein assays (Bio-Rad, BioRad DC; Mississauga, ON). Assay compatibilities were determined by the dithiothreitol (DTT) and SDS contents of the lysing buffers in accordance with manufacturer's instructions.

For specific enrichment of proteins in the Kit size range (98 kD-160 kD), certain sample lysates were further concentrated. This was accomplished using microconcentrator kits (Microcon-30/Centricon-30; Amicon, Inc., Beverly, MA) with a molecular weight cut-off of 30 kD. All solutes larger than 30 kD were retained on a low-binding membrane and were subsequently recovered into a fresh vial.

10.2 Electrophoresis and Western Blotting of Protein Samples

Proteins were separated on a denaturing one-dimensional acrylamide gel consisting of a bottom layered resolving gel {5-10% bis-acrylamide, 1.5 M Tris pH 8.8, 10% SDS, TEMED and 10% aprotinin sulfate) and a top-layered 4% stacking gel (4% bis-acrylamide, 0.5 M Tris pH 6.8, 0.4% SDS, TEMED and 10% aprotinin sulfate) in a vertical electrophoresis system containing 1X running buffer (Tris, glycine pH 8.3, SDS). Gels were

transferred overnight at 4^0C to a nitrocellulose membrane (Hybond C Extra, Amersham, Arlington Heights, IL) in circulating transfer buffer.

All subsequent immunoblotting was performed at RT in a humidified chamber with shaking. Following blocking for 1 h in TBST (180 mM NaCl, 10 mM Tris, 0.05% Tween 20) containing 5% skim milk, the blots were washed with TBST, and incubated with one of the following antibodies: i) a mouse anti-Kit (human) monoclonal antibody (Boehringer Mannheim #1428 616) at 0.1-0.5 μg/ml, or ii) a polyclonal anti-Kit antiserum (Ab-1; Oncogene Sciences, Uniondale, NY). The secondary antibody used was a horseradish peroxidase (HRP) conjugated goat anti-mouse IgG heavy and light chain antibody at a dilution of 1:5000 (BioRad, Mississauga,Ont.).

Visualization of protein bands was accomplished using an enhanced chemiluminescence (ECL) detection system (LumiGLO; Kirkegaard and Perry Laboratories, Gaithersburg, MD). Briefly, blots were exposed to the chemiluminescent reagents for 1 min, and then immediately exposed to autoradiographic film for 30s-10 min.

11. Establishment of Primary Ovarian Carcinomas in Culture

11.1 Retrieval, Handling and Establishment of ovarian tumours *in vitro*

Ovarian tumour explants from women undergoing gynecological surgery were transferred from the operating room to the pathology lab, placed into defined media (see below) and taken to the research laboratory immediately following removal. All subsequent handling of the tissues was performed under sterile conditions in a laminar flowhood. Tissues were minced with sterile blades into small (2 - 4 mm) pieces, of which two smaller pieces were removed, frozen in liquid nitrogen, wrapped in aluminum foil, and stored at -80^{0}C for later mRNA and/or protein analysis. The remaining pieces were digested (usually overnight) in a collection media solution (OSE media; see below) supplemented with 1000 units of pure (Collagenase Type XI; Sigma) and crude (Worthington Biochemical Corporation, Freehold, NJ) collagenases in a ratio of 1:4. Following digestion, the aggregates were dissociated by gentle pipetting, and undigested material was sedimented for several hours. The supernatant was decanted and spun at 3000 rpm for 5 min and then resuspended into media, while the sediment was resuspended directly without spinning. The tumours were collected and the cells were grown in media containing 50:50 medium 199 (Sigma): medium 105 (MCDB 105; Sigma) and 50 μg/ml gentamicin (Gibco BRL), with the additional supplementation of 10% heat-inactivated FCS upon plating (OSE media; Lounis *et al.*, 1994). In later attempts (N3 - N11), duplicate cultures were also established in another media formulation (E3 media) containing reduced concentrations of FCS and additional growth factors {DMEM-F12 (Gibco BRL), 5 x 10^{-5} M each ethanolamine and O-phosphorylethanolamine, 5 ng/ml EGF, 5 μg/ml insulin, 10 μg/ml transferrin, 0.05 mg/ml gentamicin and 3 % FCS}. Reduction of medium

serum concentration has been described as one means of limiting fibroblast contamination in primary cultures of epithelial origin (Hirte *et al.*, 1994). Several other modifications to the original protocol were also made in order to optimize the establishment of cell cultures from ovarian tumours. An alternate method of collection was introduced in which tumour pieces were placed directly into sterile media in the operating room, and not routed through the less sterile environment of the pathology lab. Additionally, some tumours were hemolytically lysed (Erythrocyte Lysis Buffer, Qiagen) following collagenase digestion to remove excess blood cells from the explant. The method of introduction into culture was also modified, such that tumour pieces were pressed directly onto tissue culture dishes in the absence of enzymatic digestion.

All dispersed cells and tumour pieces were initially seeded into 35 mm dishes, and then split once confluent into consecutively larger dishes. Established cultures were analysed for expression of Kit protein and/or keratin, either by Western analysis (Kit; see section **10**) or by immunocytochemistry (Kit and keratin; section **11.2**).

11.2 Immunolocalization of Keratin and Kit in Cultured Ovarian Tumour Cells

Cultured primary tumour cells were harvested by trypsinization (0.025% trypsin/ 1 mM EDTA in PBS) and centrifuged at 3000 rpm for 3-5 min. Cells were resuspended in appropriate media and seeded at approximately 50,000 cells/dish on 0.15% gelatin coated coverslips in 35 mm dishes. Upon reaching the desired confluence (50-70%), coverslips were briefly rinsed twice in Stockholm's PBS (S-PBS; pH 7.4), and then cells were fixed in cold

methanol for 5 min at -20^{0}C. Coverslips were allowed to air dry for 5 min prior to rehydration in S-PBS (15 min).

Cells were incubated for 1 h at RT in a humidified chamber with one of two primary antibodies: i) a polyclonal anti-Kit antibody (Ab-1) or ii) an anti-Keratin AE1/AE3 antibody (Boehringer Mannheim) at 1:50 in 1X S-PBS, 1% BSA, 0.1% sodium azide and 0.2% Triton X. AE1/AE3 is a pooled monoclonal antibody (mAb) which recognizes 11 of the possible 19 epithelial keratins: all of the high-molecular weight epithelial keratins (56-67 kD) as well as most of the low-molecular weight keratins (40, 48, 50, 50' and 52 kD). Coverslips were rinsed (3 x 15 min) in 1X S-PBS and then treated for 1 h at RT with a 1:100 dilution of biotinylated anti-mouse IgG (Amersham Life Science) in S-PBS. Following 3 additional washes in S-PBS, bound antibody was visualized with a 1:50 dilution of fluorescein (FITC)-conjugated streptavidin (Amersham Life Science) in S-PBS for 30 min. Coverslips were rinsed 3 final times, and inverted with antifade mounting medium (1 mg/ml p-phenylendiamine, 10% glycerol, 0.9% NaCl) onto perma-frost microscope slides. Control immunostaining was done by omitting the primary mAb from the procedure, and no staining was evident in any of these slides. LC-80 cells, which are known to express abundant Kit protein detectable by immunocytochemistry (Sekido *et al.*, 1993), were used as a positive control for Kit expression.

12. Statistical Analyses

All cell counts are expressed as mean ±standard error of the mean (SEM) of at least 9 values derived from 3 independent experiments. The probability of significant differences upon comparison of only two groups was determined by student's *t*-test (two-tailed) where significance was inferred at $p<0.05$. When multiple treatment groups were analysed, statistical comparisons were made by analysis of variance (ANOVA). Bonferroni's post-test was used to determine significance ($p<0.05$) between specific treatment groups when whole group differences were detected by ANOVA. All tests were performed using GraphPad Prism software (Version 2.0, GraphPad Software, San Diego, CA).

RESULTS

During this research project the possible regulation by growth factors, hormones, and cAMP of ovarian cancer cell proliferation and expression of c-*kit* and KL was examined. HEY and OVCA 429 cell lines were used as *in vitro* models for ovarian carcinomas, and were cultured under different conditions with E_2, P_4, T, FSH, hCG, EGF, TGF-α, TGF-β, dbcAMP, 8-bromo-cAMP and cholera toxin. Cellular growth, mRNA and protein expression following exogenous stimulation with these compounds were assessed using hemocytometer counts and Northern and Western blotting. The two cell lines were chosen because they co-express c-*kit* and KL; thereby having potential for autocrine stimulation of Kit by KL, and the possible opportunity to manipulate expression levels. Additionally, primary ovarian tumours were established in culture and assessed for c-*kit* mRNA expression, as a possible means of studying an even more representative model of ovarian cancer.

13. Regulation of HEY Cells by Growth Factors

13.1 Growth Factor Effects on Proliferation

The effects of EGF, TGF-α and TGF-β on HEY cell proliferation were assessed under different culture conditions. HEY cells were cultured in the presence or absence of each of the above growth factors in 10% or 1% FCS-containing media, or in FCS-free media. Results from culture of HEY cells with EGF, TGF-α and TGF-β under varying FCS conditions are depicted in Figures 2-4, respectively. In all cultures examined, there were no significant changes in HEY cell growth mediated by EGF, or TGF-α/β compared to control in any of the FCS-containing conditions.

Initial experiments were completed in 10% FCS, however, when no response was observed with either TGF-α and EGF, it was suspected that the presence of the FCS might be masking the effects of exogenously added growth factors. Figure 2 depicts the effects of EGF on HEY cell proliferation in 10% FCS. After 48 h, the total number of untreated HEY cells was $41.0 \pm 3.1 \times 10^4$ (n =9), which was not different from the numbers of EGF-treated cells ($39.2 \pm 1.3 \times 10^4$). Similarly, there were no significant changes in HEY cell proliferation following treatment with either TGF-α or TGF-β ($44.9 \pm 3.2 \times 10^4$ and $46.0 \pm 2.8 \times 10^4$ cells respectively, versus $47.0 \pm 4.2 \times 10^4$ untreated cells). Therefore, attempts were made to maintain HEY cells in FCS-free media in order to remove any potential activity of FCS in masking the effects of added growth factors. At the time points examined (EGF: 48 h; TGF-α and TGF-β: 24 h), HEY cells did not tolerate FCS-depleted conditions. After less than 24 h in culture, many of the untreated HEY cells had become rounded, and lifted from the surface of the tissue culture dish, and most of these cells were not viable (as assessed by trypan blue and visual observation). Furthermore, determination of final cell numbers indicated that cells cultured in FCS-free media (regardless of plating in FCS-containing or FCS-depleted media), with or without added growth factors, did not maintain even the initial seeding density (dashed line on Figures 2-4 mark initial seeding density = 5×10^4 cells).

Therefore, because these HEY cells did not survive FCS-free conditions, subsequent experiments were completed using 1% FCS in the culture media, and cells were seeded at a higher initial seeding density (7.5×10^4 cells). Compared to the FCS-free conditions, all cell groups did much better in 1% FCS than in FCS-free, however, none of the added growth factors elicited any changes in HEY cell proliferation even under these modified conditions.

FIGURE 2: ***Effects of EGF on HEY Cell Proliferation***

Cells were cultured in the presence or absence of 10 ng/ml EGF for 48 h in media containing varying amounts of FCS (10, 1, and 0%), and final cell counts were determined using a hemocytometer. Values are presented as mean ± SEM where each data point was derived from at least 3 replicates of 2 or more experiments (10% and 1%: n = 9; 0%: n = 6). The dashed line represents the initial seeding density of 5.0×10^4 cells/dish.

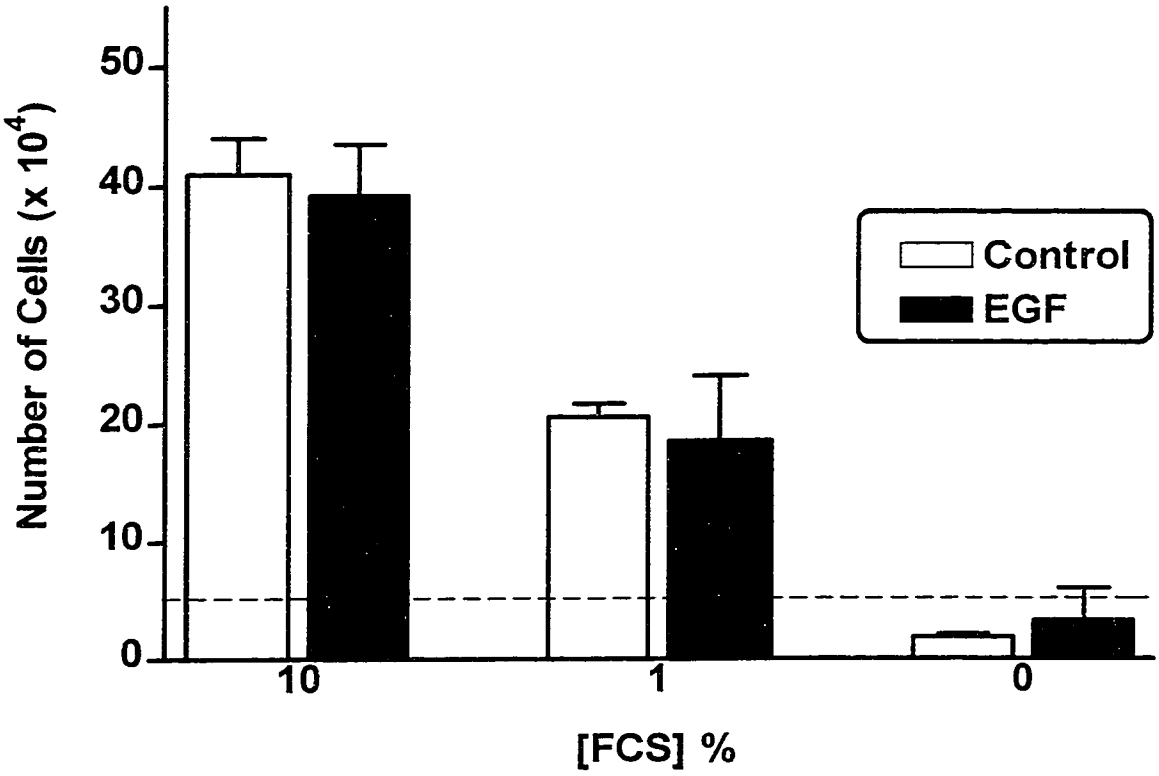

Number of Cells (x 10^4)
50
40
30
20
10
0
10
1
0
[FCS] %
Control
EGF

FIGURE 3: ***Effects of TGF-α on HEY Cell Proliferation***

Cells were cultured in the presence or absence of 10 ng/ml TGF-α in media containing varying amounts of FCS (10, 1, and 0%), and final cell counts were determined using a hemocytometer. Values are presented as mean ± SEM where each data point was derived from at least 3 replicates of 3 individual experiments (n = 9 for each treatment group). The dashed line represents the initial seeding density of 5.0×10^4 cells/dish.

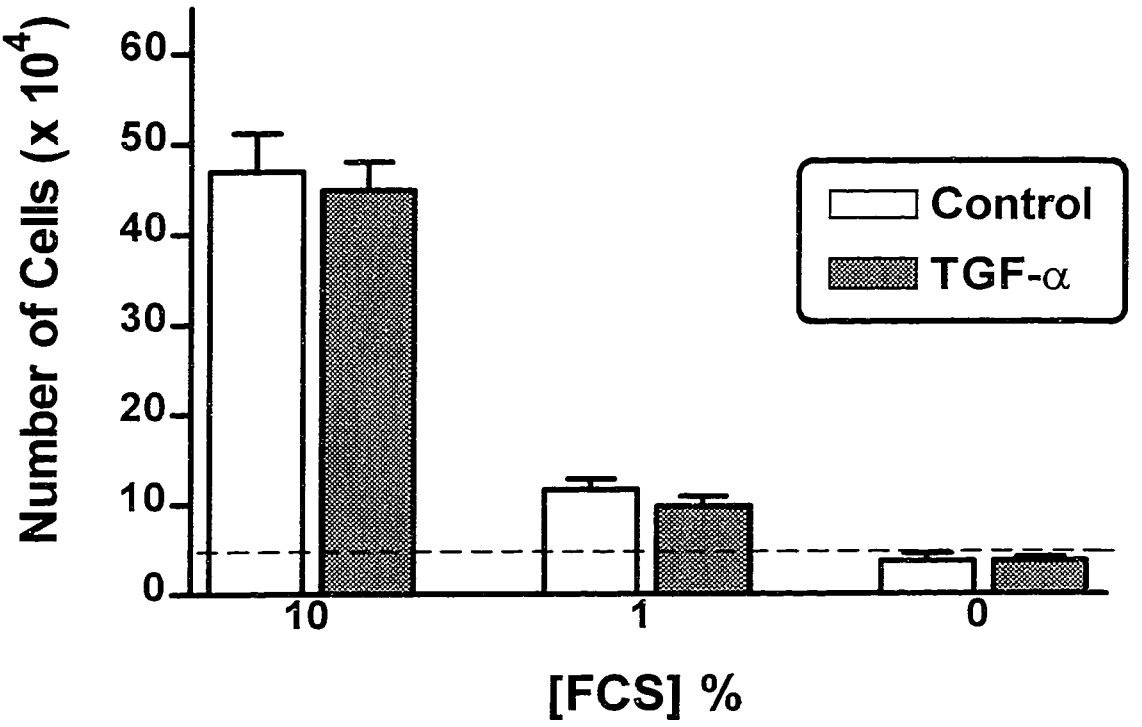
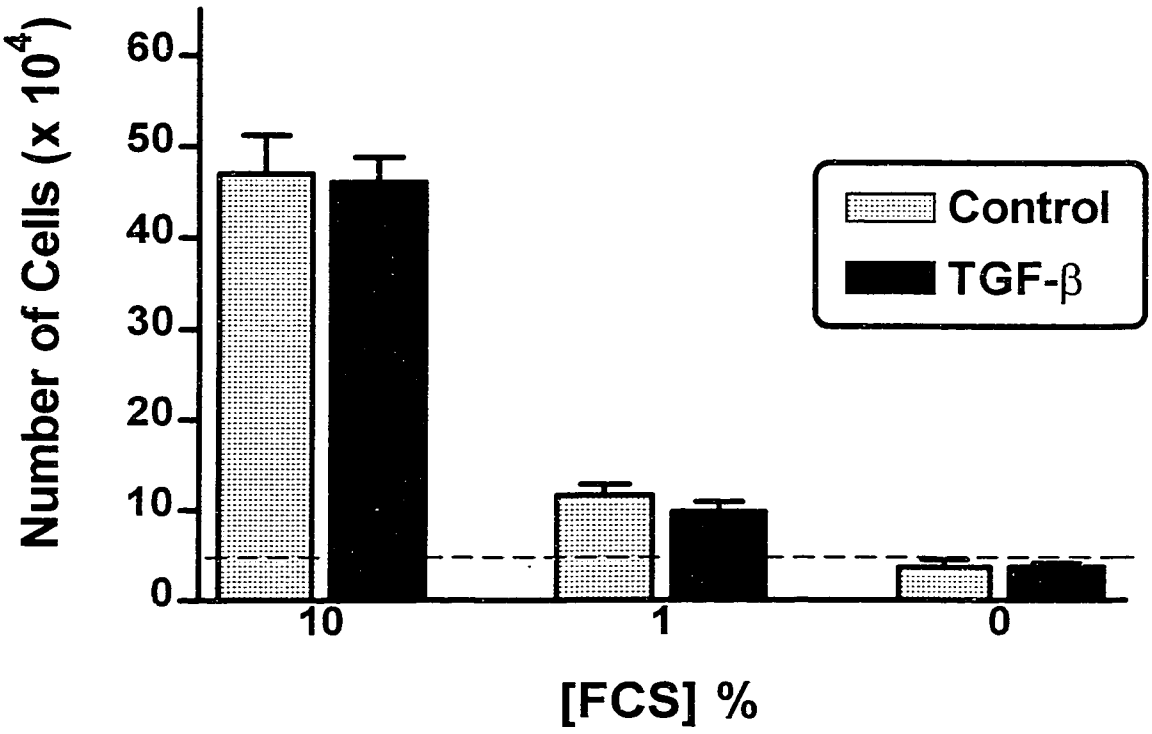

Number of Cells (x 10^4)
60
50
40
30
20
10
0
10
1
0
[FCS] %
Control
TGF-α

FIGURE 4: ***Effects of TGF-β on HEY Cell Proliferation***

Cells were cultured in the presence or absence of 10 ng/ml TGF-β in media containing varying amounts of FCS (10, 1, and 0%), and final cell counts were determined using a hemocytometer. Values are presented as mean ± SEM where each data point was derived from at least 3 replicates of 3 individual experiments (n = 9 for each treatment group, except 10% TGF-β, n = 8). The dashed line represents the initial seeding density of 5.0×10^4 cells/dish.

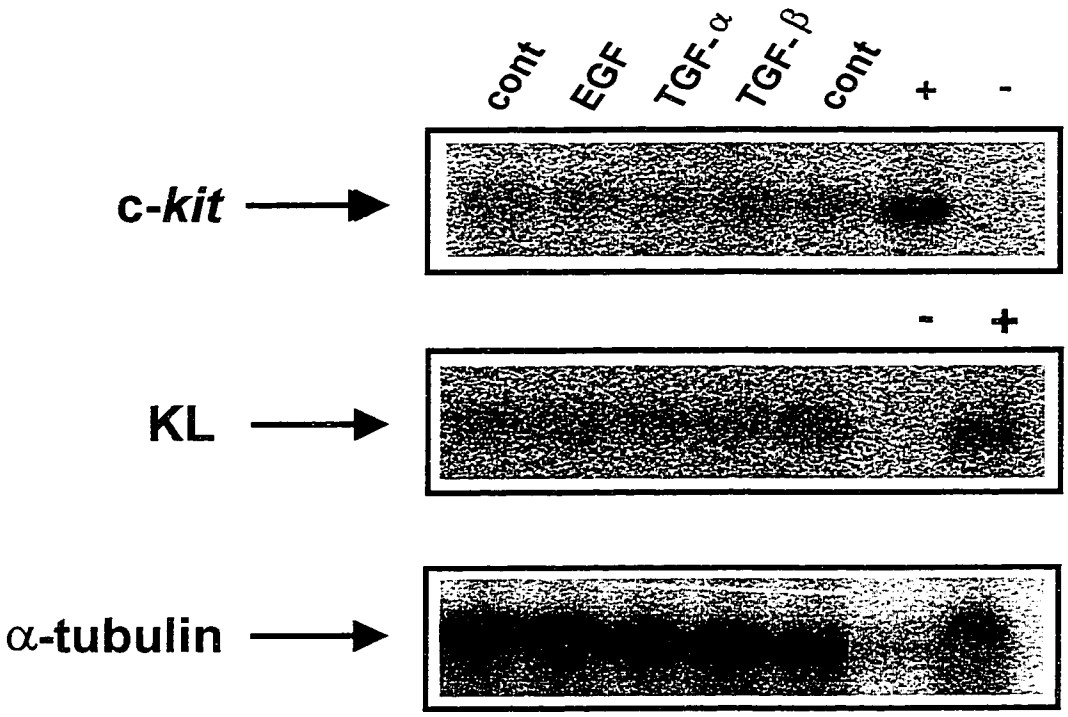

Number of Cells (x 10^4)
60
50
40
30
20
10
0
10
1
0
[FCS] %
Control
TGF-β

13.2 Growth Factor Effects on c-*kit* and KL Expression

Following examination of the effects of the growth factors EGF, TGF-α and TGF-β on HEY cell proliferation, analysis of c-*kit* and KL mRNA expression in these cells was performed using Northern blotting. Figure 5 depicts the expression of c-*kit* and KL mRNA transcripts in HEY cells cultured in 10% FCS-containing media. This ovarian carcinoma cell line expresses appreciable baseline levels of c-*kit* and KL mRNA. No change was detected in the steady-state levels of either transcript relative to control in response to any of the added growth factors, as determined by densitometry.

FIGURE 5: ***Northern Blot Analysis of c-kit and KL in HEY in Response to Growth Factors***

Northern analysis was performed on 15 μg of total RNA extracted from HEY cells cultured for 48 h in the presence or absence of 10 ng/ml EGF, TGF-α or TGF-β, in 10% FCS-containing media. RNA was separated on a 0.9% agarose gel, transferred to a nylon membrane and first hybridized to a ^{32}P-labelled cDNA probe containing full-length human c-*kit*, stripped and then reprobed using a ^{32}P-labelled KL cDNA fragment. LC-80 (c-*kit* +) small cell lung cancer and OV2008 (KL+) ovarian carcinoma RNA samples were included to serve as combined positive and negative controls. LC-80 RNA was deliberately underloaded (5 μg versus 15 μg) because of its extremely strong *c-kit* signal. This blot is representative of three experiments.


cont
EGF
TGF-α
TGF-β
cont
+
-
c-kit
-
+
KL
α-tubulin

14. Hormonal Regulation of HEY and OVCA 429

14.1 Effects on HEY Cell Proliferation

To determine the effects of exogenous hormones on ovarian carcinoma cell proliferation, HEY cells were cultured in the presence or absence of various steroids (E_2, P_4 or T) or gonadotropins (FSH and hCG). After 48 h, the total number of untreated HEY cells was 40 ± 2.5 x 10^4 cells (n = 16), 8-fold greater than the 5 x 10^4 cells which were initially seeded (Figure 6). There were no significant changes observed in cell morphology or rates of proliferation relative to control in any of the treatment groups (Figure 6). While there was a tendency for a mild growth inhibitory effect following culture of HEY with E_2 or P_4 (E_2 = 33.6 ± 2.5 x 10^4 cells, n = 9; P_4 = 32.4 ± 2.6 x 10^4cells, n = 8), these differences were not statistically different from control.

14.2 Effects on OVCA 429 Cell Proliferation

A parallel series of experiments was completed on another ovarian carcinoma cell line, OVCA 429 cells, which also express c-*kit*. These cells display a similar lack of effect on proliferation in response to hormonal stimulation (Figure 7). Owing to an inherently slower growth rate, the final cell number reached was approximately one-third less than that of HEY cells (24.5 x 10^4 versus 40 x 10^4 cells), but the lack of growth-modulating effects induced by exogenous steroids or gonadotropins in OVCA 429 cultures was comparable to that observed in the HEY studies. While there was a trend towards growth inhibition of OVCA 429 cells following FSH or hCG stimulation (20.0 ± 1.2 x 10^4 cells, n = 11 and 19.7 ± 1.1 x 10^4 cells, n = 11, respectively, versus control 24.5 ± 1.9 x 10^4 cells (n =16)}, these differences did not

attain significance.

In an effort to eliminate the possibly confounding effects of steroids present in the FCS on the above hormonal regulation experiments, further studies were completed with OVCA 429 cells in charcoal-treated FCS. OVCA 433 cells, which are known to possess estrogen receptors were used as a positive control for the stimulatory effect of E_2 in charcoal-treated FCS (Figure 8A). While the growth of OVCA 433 cells was significantly increased in the presence of 500 and 5000 nM E_2, (500 nM: 28.1 ± 1.3 x 10^4 cells and 5000 nM: 25.0 ± 1.9 x 10^4 cells, versus control: 14.4 ± 0.6 x 10^4 cells; $p < 0.001$), the addition of exogenous E2 to OVCA 429 cells cultured in charcoal-treated FCS-containing media did not alter OVCA 429 cell growth (Figure 8B).

FIGURE 6: ***Effect of steroid and gonadotropic hormones on HEY ovarian carcinoma cell proliferation***

HEY cells were seeded at 5 x 10^4 cells/dish in medium with 10% FCS and cultured in the presence or absence of 500 nM each E_2, P_4 or T, 120 ng/ml FSH or 5 IU/ml hCG. Cell number after 48 h was determined by two independent counts using a hemocytometer. Values represent mean +/- SEM of viable cells from three independent experiments (control, n = 15 replicates; all other groups, n = 9).

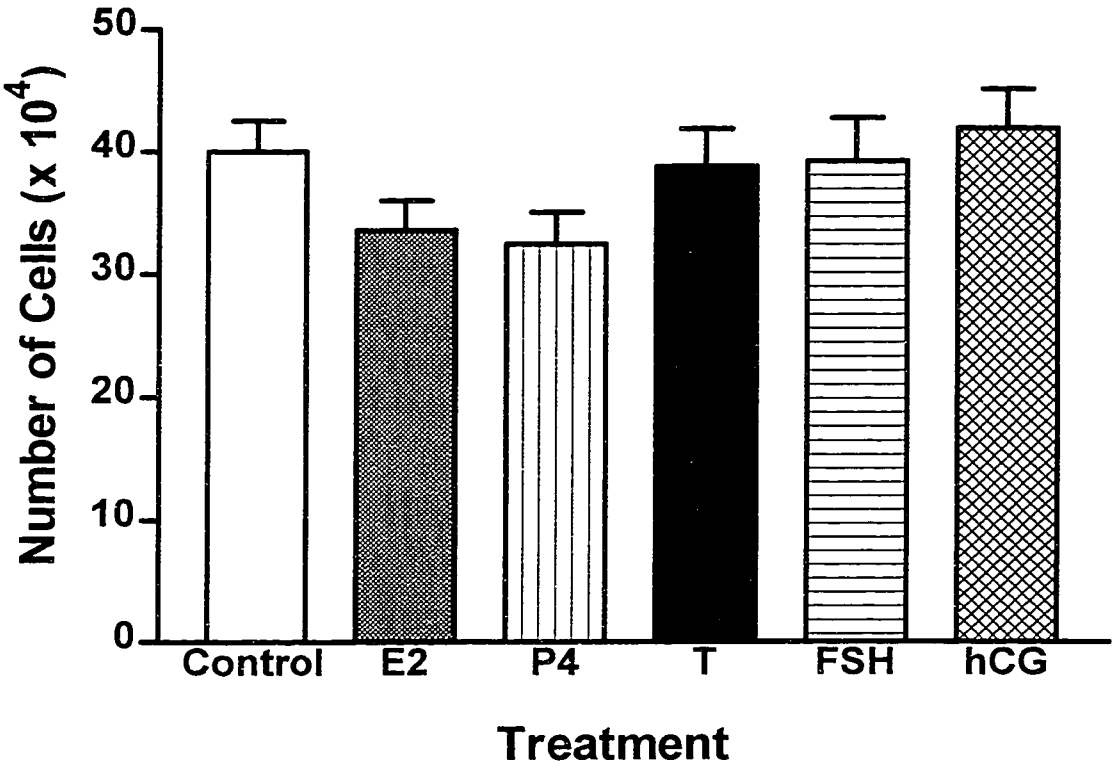

Number of Cells (x 10^4)
50
40
30
20
10
0
Control
E2
P4
T
FSH
hCG
Treatment

FIGURE 7: ***Effect of steroid and gonadotropic hormones on OVCA 429 cell proliferation***

OVCA 429 cells were seeded at 5 x 10^4 cells/dish in medium with 10% FCS and cultured in the presence or absence of 500 nM each E_2, P_4 or T, 120 ng/ml FSH or 5 IU/ml hCG. Cell number after 48 h was determined by two independent counts using a hemocytometer. Values represent mean +/- SEM of viable cells from three independent experiments (control: n = 16; steroids: n = 9; and gonadotropins: n =11 replicates).

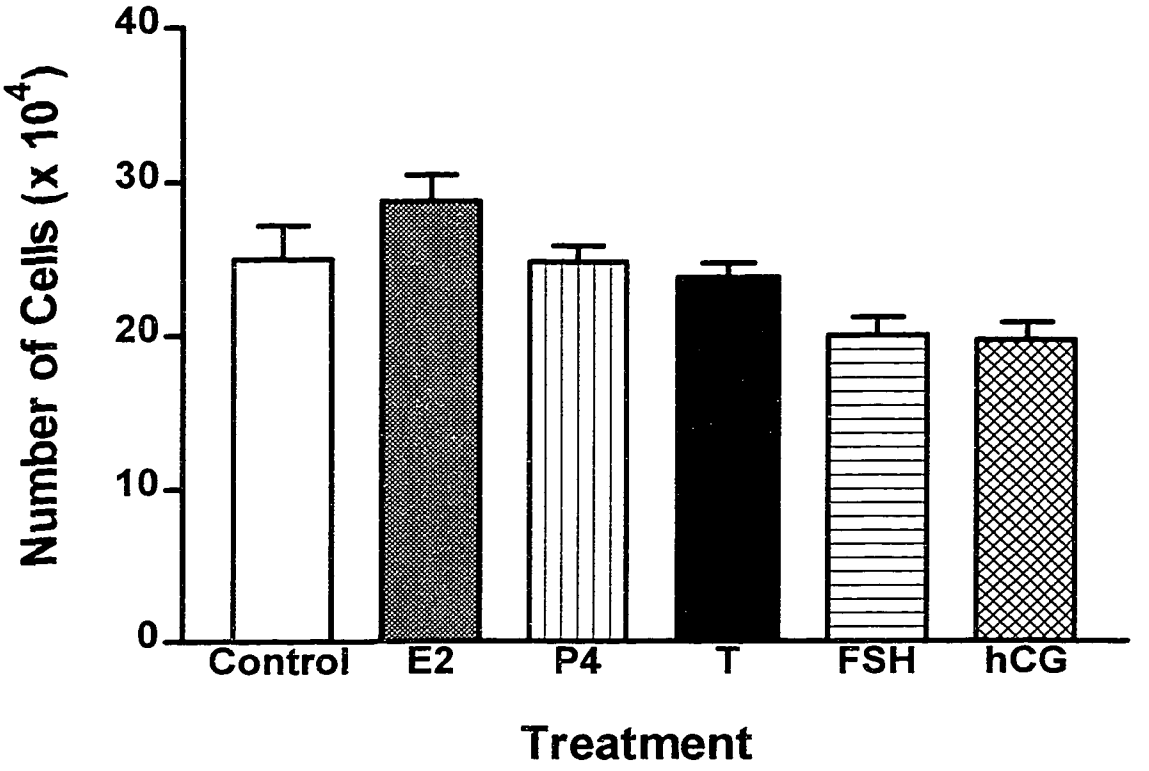
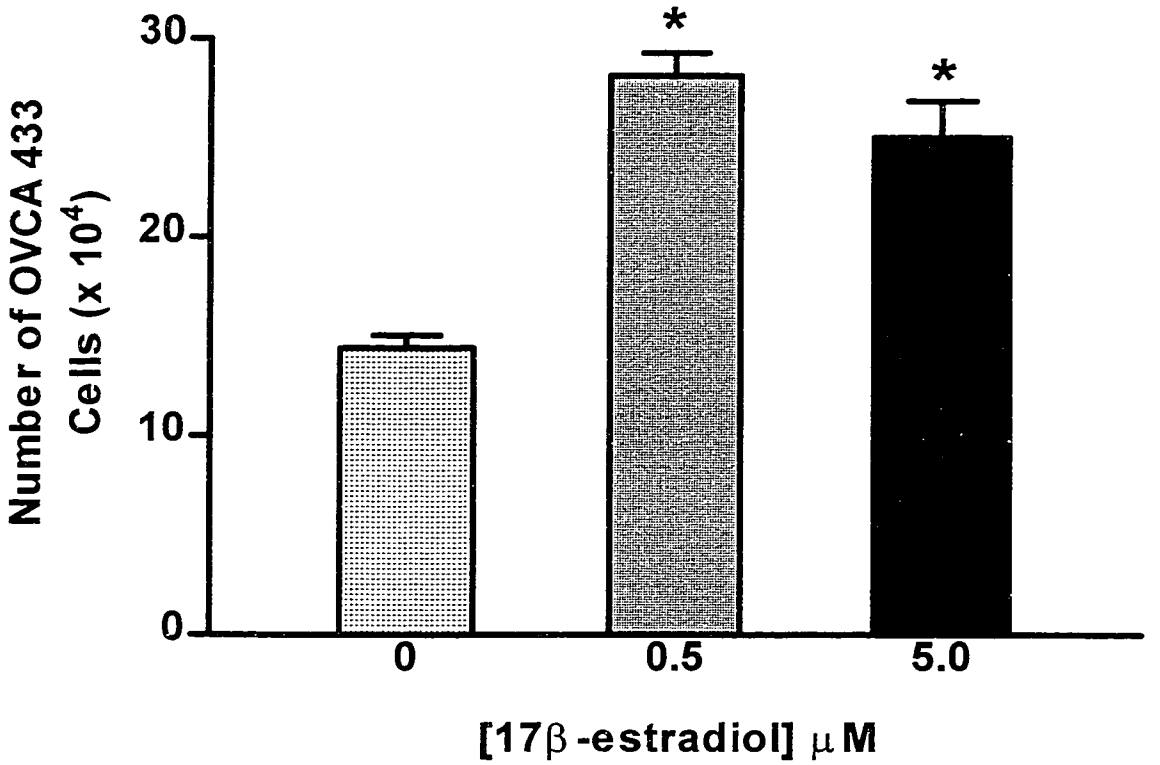

Number of Cells (x 10^4)
40
30
20
10
0
Control
E2
P4
T
FSH
hCG
Treatment

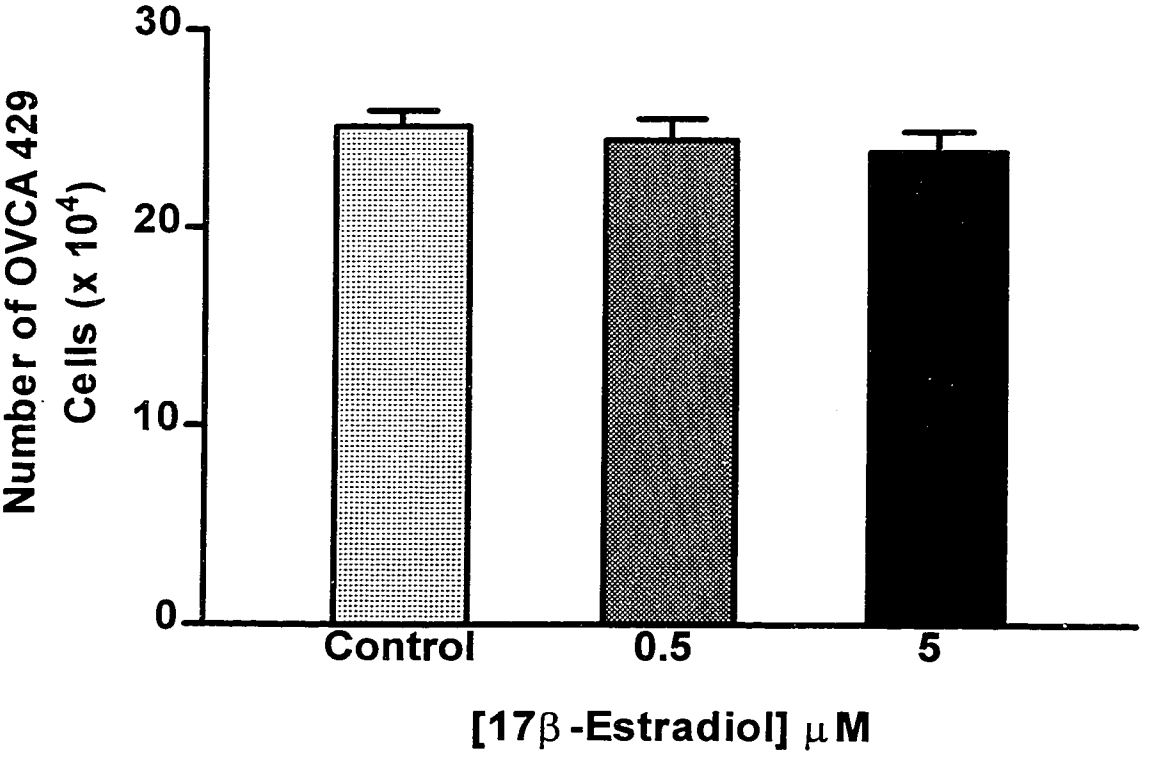
FIGURE 8: Effect of charcoal-treated FCS on OVCA 433 and OVCA 429 cell proliferation

OVCA 433 (A) and OVCA 429 (B) cells were seeded at 7.5 x 10^4 cells/dish in medium containing 10% charcoal-stripped FCS. Cells were cultured for 48 h in the presence or absence of 500 or 5000 nM E_2. Cell number was determined by two independent counts using a hemocytometer. Values represent mean ± SEM from four independent experiments (n = 12 replicates for all groups).

A


30
20
10
0
Number of OVCA 433 Cells (x 10^4)
*
*
0
0.5
5.0
[17β-estradiol] μM

B


30
20
10
0
Number of OVCA 429 Cells (x 10^4)
Control
0.5
5
[17β-Estradiol] μM

14.3 Hormonal Effects on Kit and KL mRNA expression

Following examination of the effects of various hormones on HEY and OVCA 429 cell proliferation, northern analysis was performed to identify any changes in the gene expression for c-*kit* and KL as a consequence of hormone stimulation. Figure 9 depicts the expression of c-*kit* and KL mRNA transcripts in HEY cells. This ovarian carcinoma cell line expresses appreciable baseline levels of KL mRNA, and slightly weaker expression of c-*kit* mRNA. However, no change was detected in the steady-state levels of either transcript relative to control in response to 48 h culture with E_2, P_4, T, FSH or hCG, as determined by densitometric analysis. Furthermore, treatment of OVCA 429 cells with the same hormones did not elicit any detectable changes in c-*kit* or KL expression (Figure 10).

FIGURE 9: ***Northern blot analysis of c-kit and KL mRNA expression in HEY cells following hormonal stimulation***

Northern analysis was performed on 15 μg of total RNA extracted from HEY cells cultured for 48 h with FSH, hCG, E_2, P_4 or T. RNA was separated on a 0.9% agarose gel, transferred to a nylon membrane and first hybridized to a ^{32}P-labelled cDNA probe containing full-length human c-*kit*, stripped and then reprobed using a ^{32}P-labelled KL cDNA fragment. LC-80 (c-*kit* +) small cell lung cancer, OVCA 433 (KL+) ovarian carcinoma and LC-73 (c-*kit* and KL negative) lung cancer human RNA samples were included to serve as positive and negative controls respectively. LC-80 RNA was deliberately underloaded (5 μg versus 15 μg) because of its extremely strong c-*kit* signal. This blot is representative of two experiments.

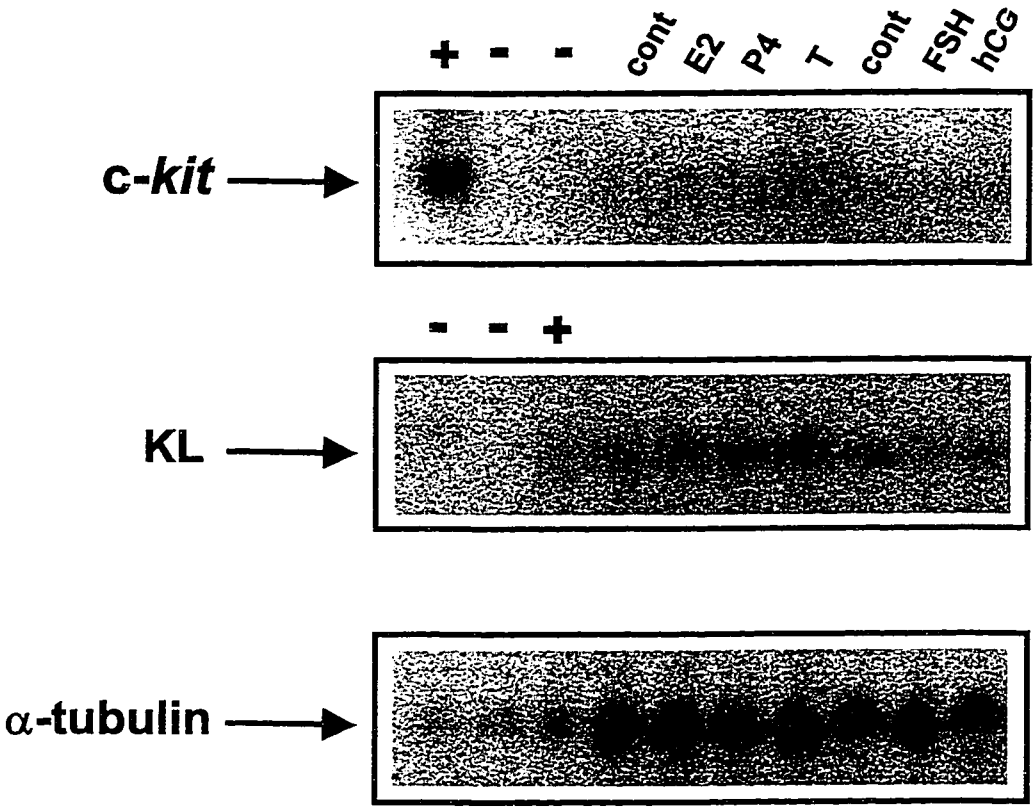
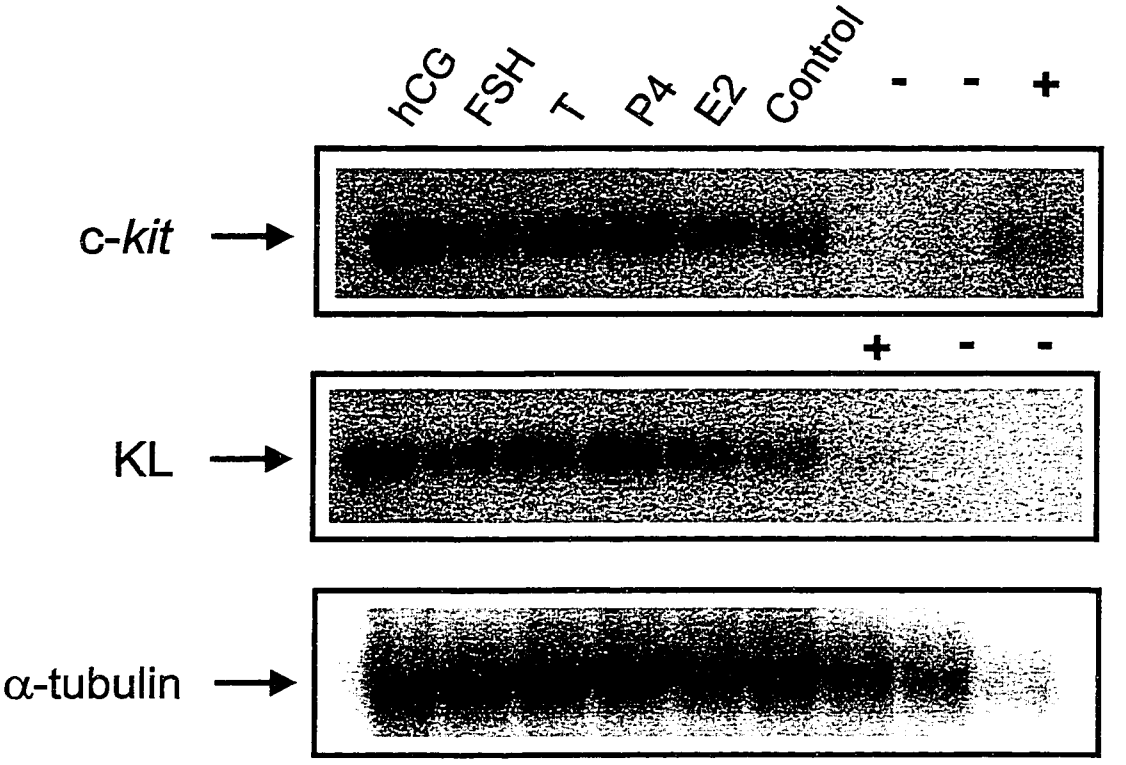

+ - - cont E2 P4 T cont FSH hCG
c-kit
- - +
KL
α-tubulin

FIGURE 10: ***Northern blot analysis of c-kit and KL mRNA expression in OVCA 429 cells following hormonal stimulation***

Northern analysis was performed on 15 μg of total RNA extracted from OVCA 429 cells cultured for 48 h with FSH, hCG, E_2, P_4 or T. RNA was separated on a 0.9% agarose gel, transferred to a nylon membrane and first hybridized to a ^{32}P-labelled cDNA probe containing full-length human c-*kit*, stripped and then sequentially reprobed using ^{32}P-labelled KL and α-tubulin cDNA fragments. LC-80 (c-*kit* +), OVCA 433 (KL+) and LC-73 (c-*kit* and KL negative) RNA samples were included to serve as positive and negative controls respectively. This blot is representative of two experiments.


hCG
FSH
T
P4
E2
Control
- - +
c-kit
+ - -
KL
α-tubulin

15. Effects of cAMP signal transduction on HEY cell proliferation, morphology and expression of c-*kit* and KL

15.1 Effects of dbcAMP and 8-bromo-cAMP on HEY proliferation

Because it has been documented that during the process of immortalization some cells may undergo gonadotropin receptor downregulation or loss (Amsterdam *et al.*, 1979), and addition of gonadotropins had not induced any significant differences in HEY cell growth, it is likely that these cells have lost gonadotropin receptor expression during immortalization. Therefore, one means of simulating membrane-bound receptor activation by exogenous ligand is to stimulate the downstream signalling pathways of those receptors instead. Due to the lack of growth regulation by both exogenously added FSH and hCG (Figures 6 & 7), and the uncertainty concerning gonadotropin receptor status in HEY cells (currently being studied in our lab), gonadotropin receptor binding was mimicked using agents which more directly elevate intracellular cAMP, the immediate downstream effector molecule of both receptors. Figure 11 demonstrates the changes in proliferation that were observed when HEY cells were cultured in the presence of increasing concentrations of dbcAMP (0 - 2 mM), a membrane-permeable cAMP analogue. After 48 h culture, untreated control cells ($n = 8$) attained a final density of $49.9 \pm 4.2 \times 10^4$ cells. In contrast, HEY cell growth was clearly inhibited by dbcAMP, and furthermore, this response was dose-dependent. While the two lower doses of dbcAMP did not affect HEY cell growth after 48h, there was significant attenuation of the growth of these cells compared to control (27.3 % inhibition, $p<0.01$) in response to 1.0 mM dbcAMP, which became even more pronounced in the presence of a two-fold higher dbcAMP concentration (2 mM; 39.9% inhibition, $p<0.001$). More potent growth inhibitory effects

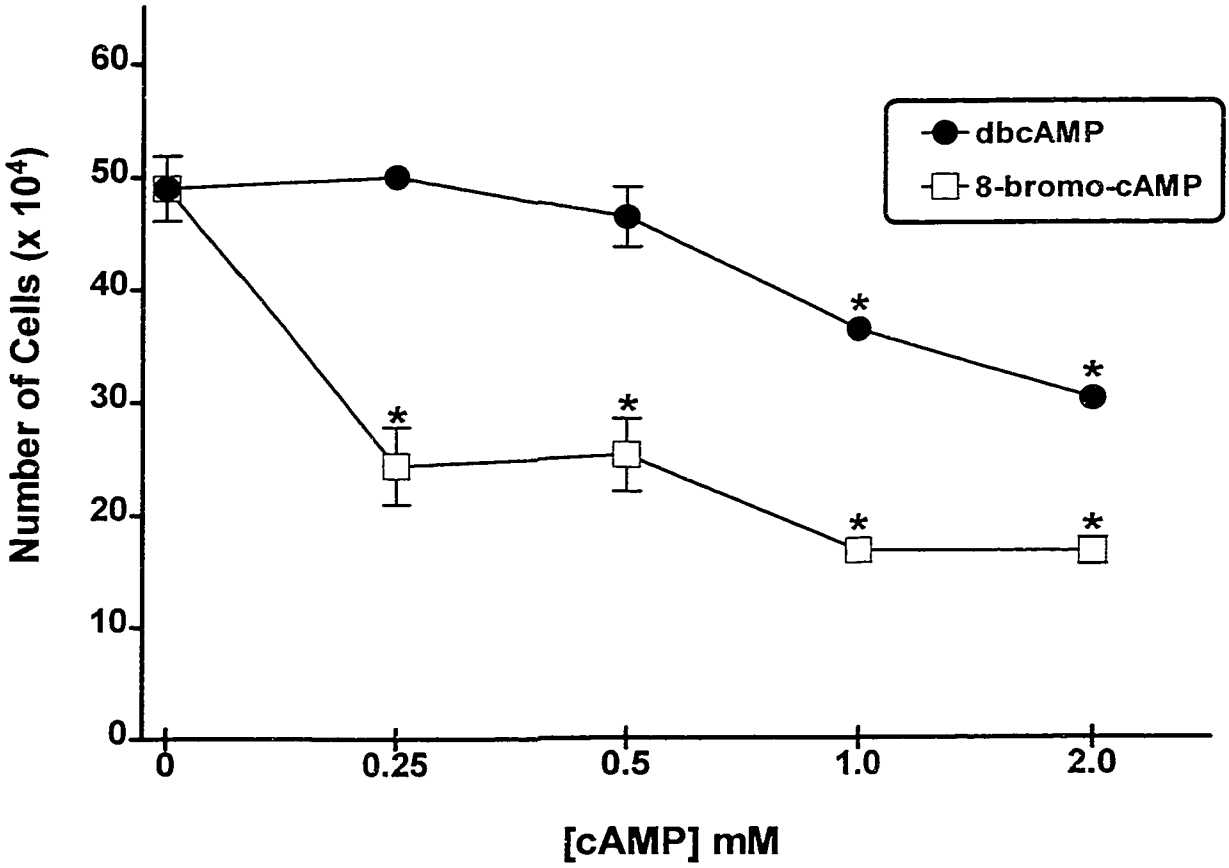
(~60% inhibition: 22.6 ± x 10^4 cells (control) versus 8.8 ± x 10^4 (2 mM dbcAMP); $p<0.001$) were observed in dbcAMP-treated OVCA 429 cells (data not shown) under comparable conditions.

As a means of confirming these dbcAMP-induced effects on HEY cell growth, a repeat set of experiments was completed using 8-bromo-cAMP as the agent responsible for elevating intracellular cAMP concentration. Unlike dbcAMP, which may be toxic to certain cell types at high concentrations (in part due to the butyryl moiety of its structure), this membrane-permeable cAMP analogue is less potentially harmful to cultured cells. Therefore, 8-bromo-cAMP was used as a means of verifying that the growth inhibition observed with dbcAMP was indeed due to elevated cAMP, and not merely a toxic effect. Like dbcAMP, culture of HEY cells with 8-bromo-cAMP also induced significant inhibitory effects on proliferation (Figure 11). In contrast, 8-bromo-cAMP treatment caused 22-44% *greater* inhibition at every concentration ($p<0.001$).

The effects of both dbcAMP and 8-bromo-cAMP were not due to toxicity since the percentage of viable cells remained high (90-97%), and was similar to control values, as determined by trypan blue dye exclusion.

FIGURE 11: ***Proliferation of HEY cells in the presence of cAMP analogues***

Cells were cultured in the presence of 0-2.0 mM dbcAMP (n = 9 for each group) or 8-bromo-cAMP (n = 6) for 48 h and final cell counts were determined using a hemocytometer. Values are presented as mean ± SEM where each data point was derived from at least 3 independent samples of 2 (8-bromo-cAMP) or 3 (dbcAMP) repeat experiments. (asterisks indicates groups which are significantly different from control; *$p<0.01$).

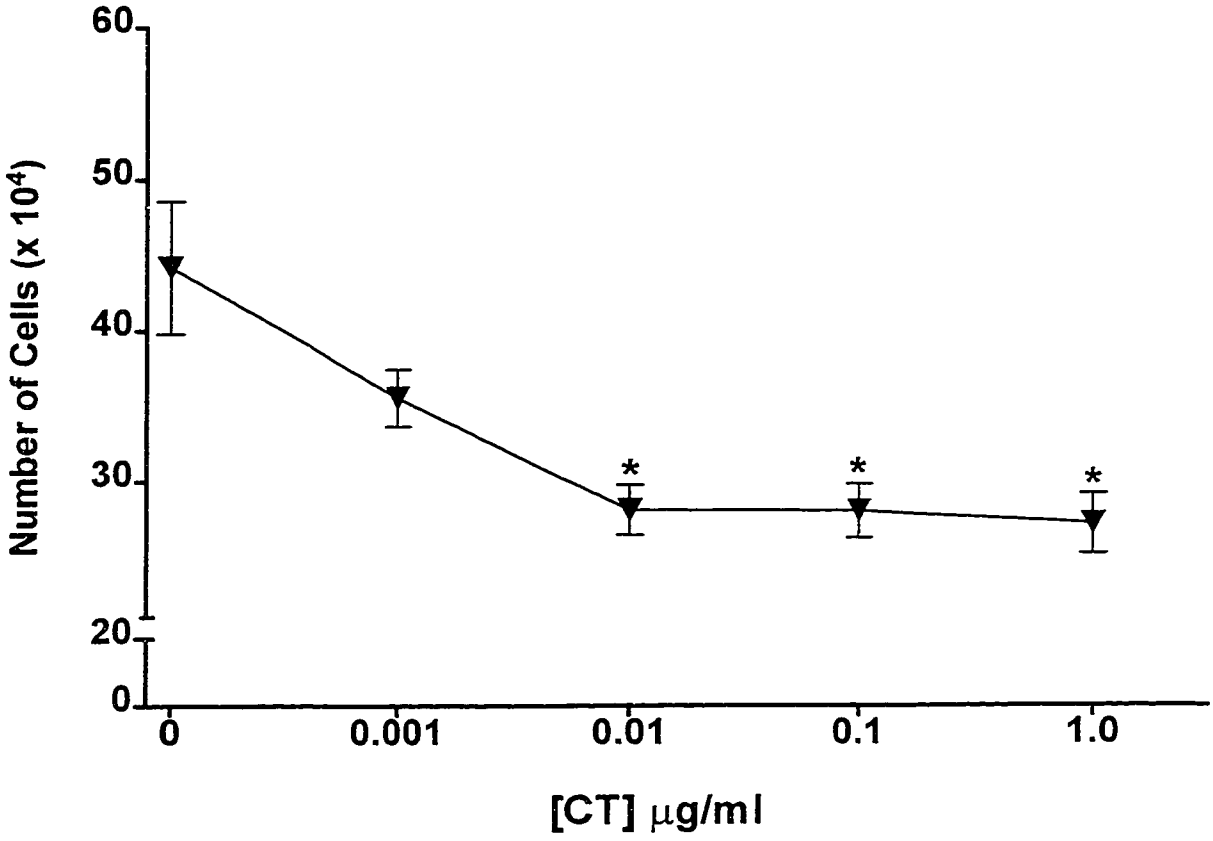

Number of Cells (x 10⁴)
60
50
40
30
20
10
0
0
0.25
0.5
1.0
2.0
[cAMP] mM
dbcAMP
8-bromo-cAMP
*

15.2 Effects of cholera toxin on HEY cell proliferation

In order to further confirm the growth inhibitory effects induced by activation of the signal transduction pathway in HEY cells, additional studies were completed using cholera toxin (CT) as an alternate stimulator of cAMP accumulation. As opposed to cAMP analogues, CT is a Gs protein activator and thereby indirectly elevates intracellular cAMP through stimulation of adenylate cyclase activity. In the presence of varying concentrations of CT, HEY cell proliferation was also markedly inhibited after 48 h culture (Figure 12). Interestingly, this growth-inhibitory effect was observed to plateau at the second lowest dose used (0.01 μg/ml; 36.4% inhibition with respect to control; $p<0.001$). Higher concentrations of CT were equally effective in suppressing HEY cell growth, but they did not induce substantially greater reductions in cell number compared to 0.01 μg/ml (36.4% and 38.5% at 0.1 and 1.0 μg/ml respectively).

FIGURE 12: ***HEY cell proliferation in the presence of Cholera Toxin***

Cells were seeded at 5 x 10^4 cells/dish and cultured in presence of 0 - 1.0 μg/ml CT for 48 h. Final cell numbers were counted using a hemocytometer and values represent mean ± SEM for at least 3 experiments with 3 independent samples per experiment. (* indicates treatment groups which are significantly different from control, $p < 0.001$).

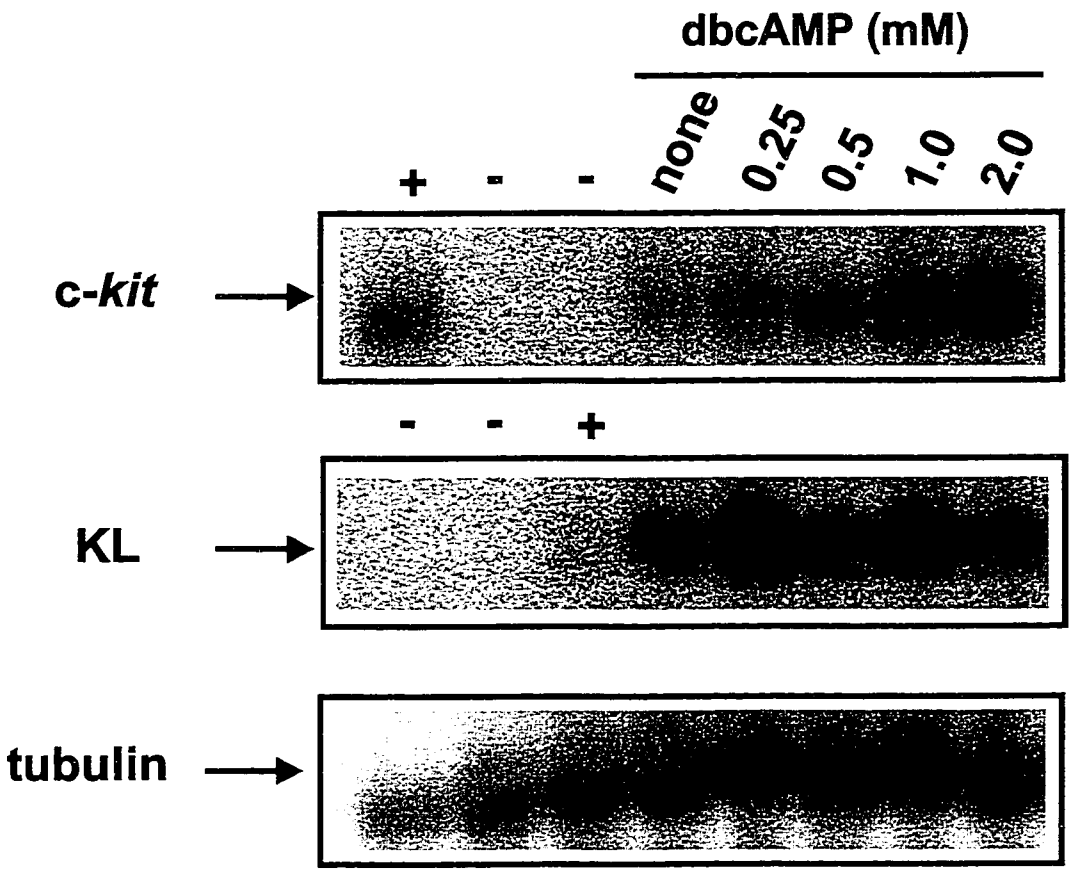

60
50
40
30
20
0
Number of Cells (x 10⁴)
*
*
*
0
0.001
0.01
0.1
1.0
[CT] μg/ml

15.3 Cyclic AMP-Regulated Expression of Kit mRNA in HEY cells

To investigate the effect of elevated cAMP on HEY cell gene expression, RNA was extracted from dbcAMP- and CT- treated cells and northern analysis was performed to detect c-*kit* and KL mRNA transcripts. Figures 13 and 14 display the northern analyses results for HEY cell responses to varying concentrations of dbcAMP or CT. Both of these molecules induced significant upregulation of c-*kit* mRNA. Interestingly, exposure of HEY cells to dbcAMP produced dose-dependent amplification of c-*kit* expression (Figure 13) which inversely correlated with the growth inhibition seen in these cells (refer to Figure 11), i.e there were step-wise increases in c-*kit* mRNA levels which paralleled the dose-dependent decreases in cell growth caused by dbcAMP. More than 4-fold greater levels of c-*kit* mRNA were detected in HEY cells cultured with 2 mM dbcAMP compared to control. Similar enhancement of c-*kit* expression was induced by CT (Figure 14) and were maximal at the second lowest dose provided, resulting in an almost 7-fold elevation of c-*kit* expression following culture with 0.01 μg/ml CT. While significant changes occurred in the levels of c-*kit*, there were no corresponding changes in the mRNA levels of KL (Figures 13 and 14), following culture of HEY cells with either dbcAMP or CT. The histograms at the bottom of each figure display the densitometric ratio of c-*kit* and KL expression signals in RNA samples from treated cells versus control (arbitrarily set to 100%), each standardized for α-tubulin internal controls.

FIGURE 13: ***Northern analysis of c-kit and KL mRNA expression in dbcAMP-treated HEY cells***

HEY cells were cultured for 48 h with varying concentrations of dbcAMP. Fifteen micrograms of total RNA extracted from each sample was separated on a 0.9% agarose gel, transferred to a nylon membrane and probed with a ^{32}P-labelled c-*kit* cDNA probe. RNA samples from LC-80 (c-*kit* +) small cell lung cancer, OVCA 433 (KL+) ovarian carcinoma and LC-73 (c-*kit* and KL negative) lung cancer cells were included to serve as positive and negative controls. Blots were stripped and sequentially hybridized with ^{32}P-labelled human KL cDNAs and then α-tubulin cDNAs. In order to identify changes in Kit/KL mRNA expression following dbcAMP treatment, densitometric analysis using ImageQuant software was used to quantify the intensity of each band. The histogram indicates the ratio of Kit and KL signals for each treated sample relative to its untreated control (arbitrarily set to 100%), and all bars have been standardized to their respective α-tubulin internal control. Each bar represents the mean of two experiments, and maximum and minimum data points are indicated by error bars.


dbcAMP (mM)
none
0.25
0.5
1.0
2.0
+ - -
c-kit
- - +
KL
tubulin

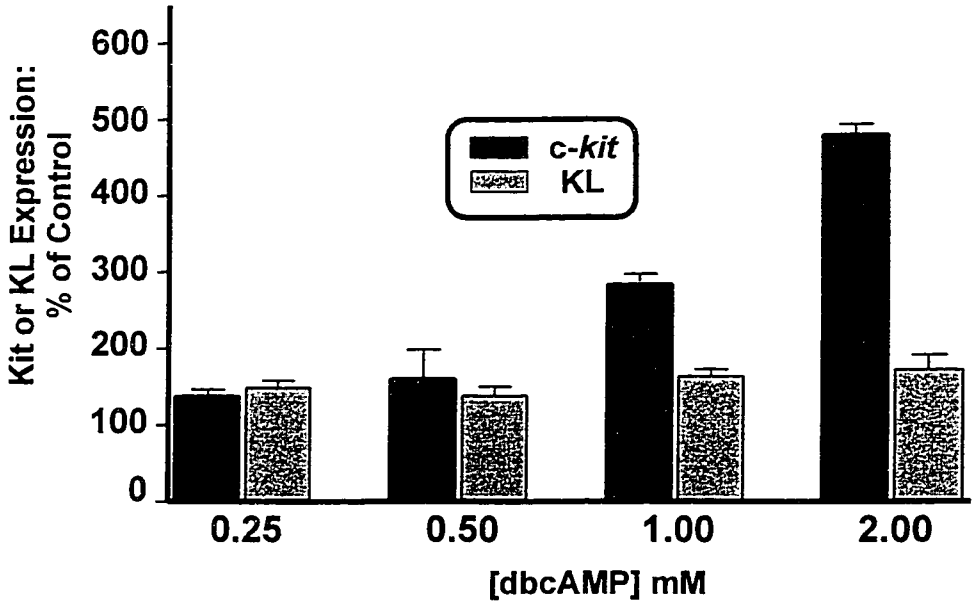
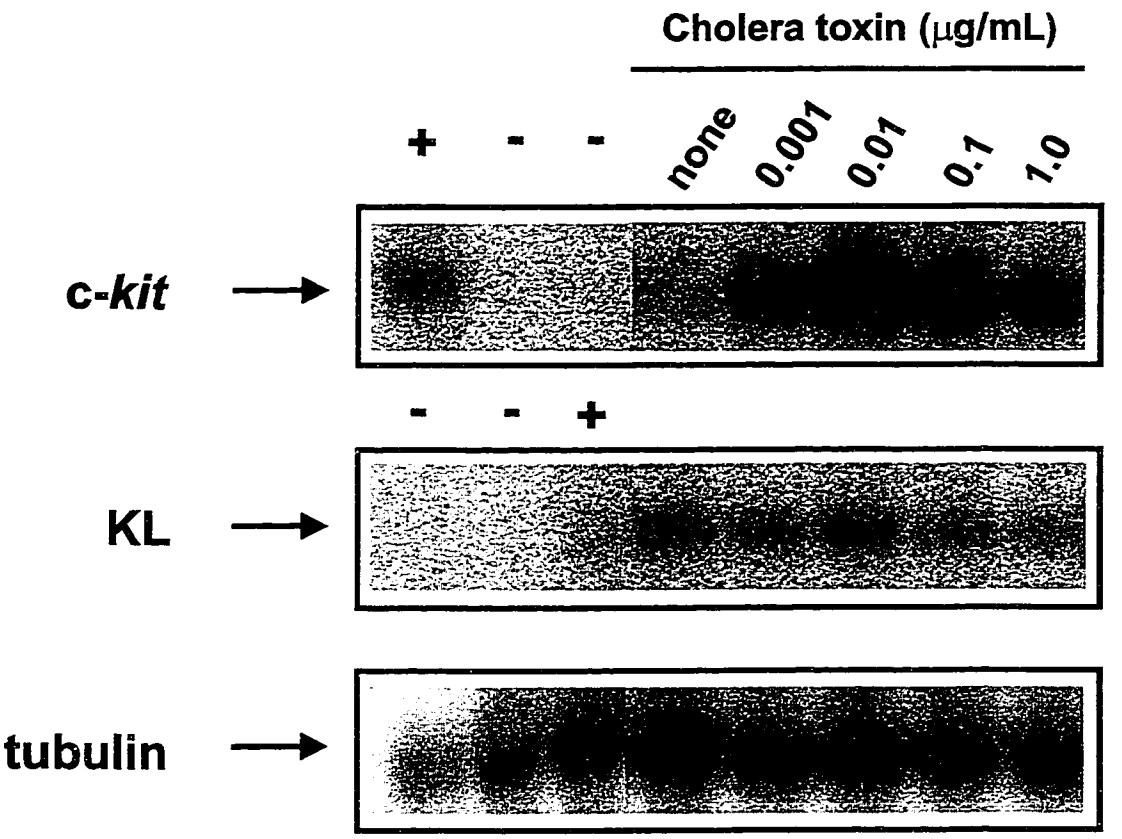
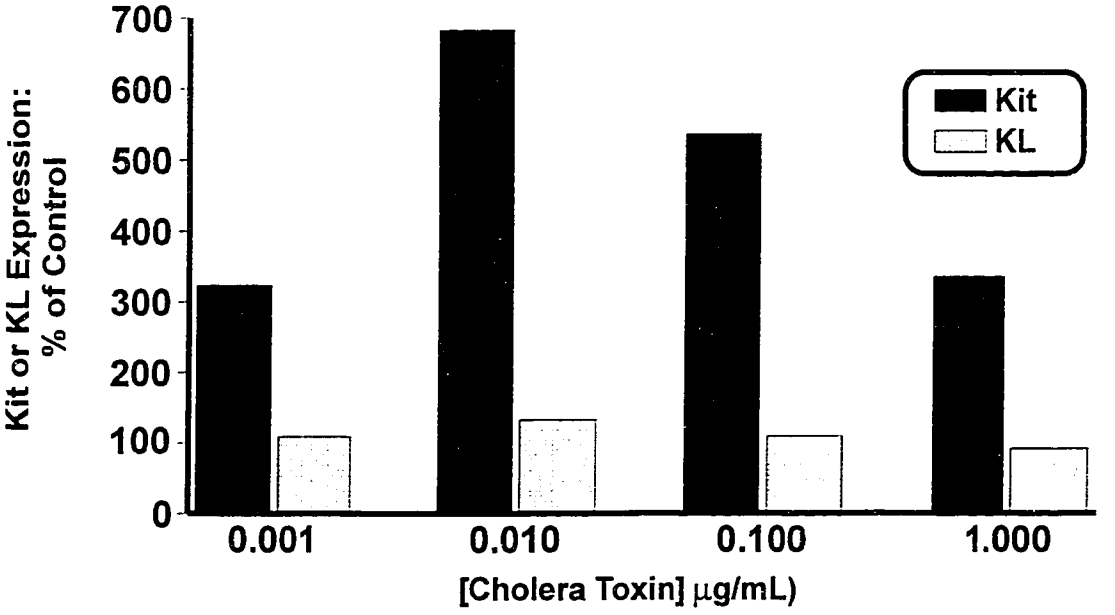

Kit or KL Expression:
% of Control
600
500
400
300
200
100
0
c-kit
KL
0.25
0.50
1.00
2.00
[dbcAMP] mM

FIGURE 14: ***Northern analysis of c-kit and KL mRNA expression in CT-treated HEY cells***

HEY cells were cultured for 48 h with varying concentrations of CT. Fifteen micrograms of total RNA extracted from each sample were separated on a 0.9% agarose gel, transferred to a nylon membrane and probed with a ^{32}P-labelled c-*kit* cDNA probe. RNA samples from LC-80 (Kit +) small cell lung cancer, OVCA 433 (KL+) ovarian carcinoma and LC-73 (c-*kit* and KL negative) lung cancer cells were included to serve as positive and negative controls. Blots were stripped and sequentially hybridized with ^{32}P-labelled human KL cDNAs and then α-tubulin cDNAs. In order to identify changes in Kit/KL mRNA expression following dbcAMP treatment, densitometric analysis using ImageQuant software was used to quantify the intensity of each band. The lower histogram indicates the ratio of Kit and KL signals for each treated sample relative to its untreated control (arbitrarily set to 100%), and all bars have been standardized to their respective α-tubulin internal control. This figure shows the results of a single experiment.


Cholera toxin (μg/mL)
+ - - none 0.001 0.01 0.1 1.0
c-kit
- - +
KL
tubulin


Kit or KL Expression:
% of Control
700
600
500
400
300
200
100
0
Kit
KL
0.001
0.010
0.100
1.000
[Cholera Toxin] μg/mL)

15.4 Cyclic AMP-Regulated Expression of Kit Protein in HEY cells

To examine the effect of dbcAMP on Kit expression, Western blotting was performed to determine if significant changes at the mRNA level led to corresponding differences at the protein level. Because dbcAMP had induced such dramatic changes in both HEY cell proliferation and c-*kit* gene expression, preliminary work was completed using protein lysates extracted from dbcAMP-treated HEY cell cultures. The culture conditions used in these studies were similar to those used previously to identify alterations in growth rate or RNA levels (section **8.0**). Briefly, protein lysates were obtained from HEY cells that had been cultured for 48 h in the presence of 0 - 2 mM dbcAMP, separated on 5-10% gels, and immunoblotted using an anti-Kit monoclonal antibody. Figure 15 is a representative blot of Kit protein expression in control and dbcAMP-treated HEY cells. Kit protein expression was abundant in the positive control lane (LC-80; ~145 and ~160 kD proteins), while a much weaker signal at ~160 kD was detected in both control HEY cell samples. However, addition of 2 mM dbcAMP significantly upregulated the level of Kit protein expression relative to basal levels, in accordance with the significant increases in signal which had been observed for c-*kit* message.

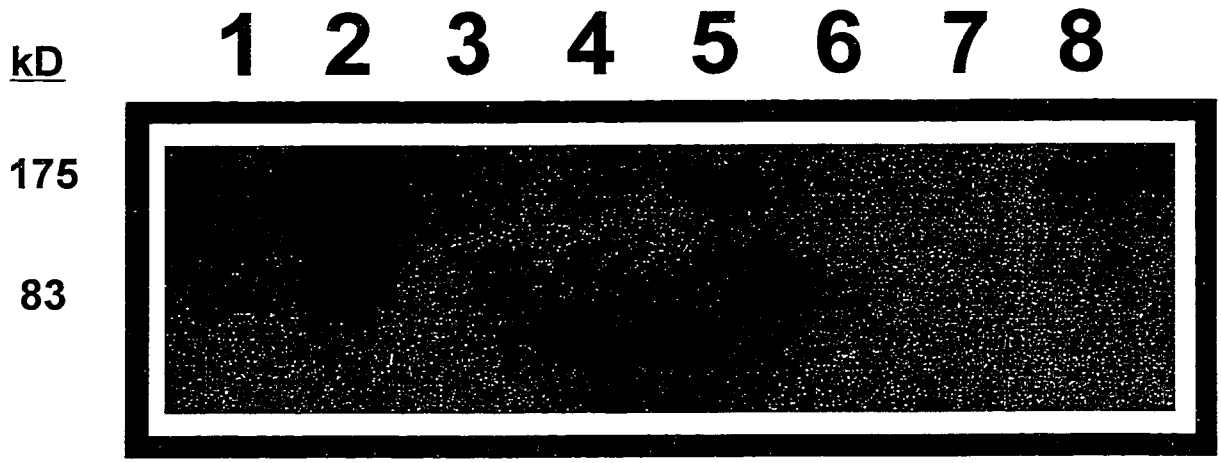
Various modifications to the protocol were tested in order to obtain a reliable assessment of baseline levels of Kit protein expression in HEY cells, and these results (Figure 15) demonstrate the strongest detectable signal in untreated cells. Other immunoblots gave similar patterns of expression, which ranged from negligible to weakly detectable (as in Figure 15). Loading varying amounts (up to 400 ng) of protein lysate, and/or concentrating the protein lysates, probing with various concentrations of anti-Kit antibody, and using a

polyclonal primary antibody did not improve the abundance of detectable Kit in untreated HEY cells (data not shown). Testing for the presence of excessive proteases in HEY cells also ruled out the possibility that the detection of protein in these cells by Western analysis was limited by excessive degradation of all proteins during the cellular lysis step (data not shown). Therefore, the expression of Kit protein in untreated HEY cells appears to be relatively low, however, this expression is significantly upregulated in the presence of dbcAMP.

FIGURE 15: ***Expression of Kit protein in HEY cells***

Protein lysates were obtained from HEY cells in 1X SDS gel-loading buffer following 48 h culture in the presence or absence of 2 mM dbcAMP. Fify nanograms of total cellular protein were separated on a denaturing agarose gel (8%), transferred to a nitrocellulose membrane and blocked with skim milk prior to immunoblotting. Proteins were detected using 0.5 μg/ml of a primary murine monoclonal anti-Kit (human) antibody, and an HRP-conjugated goat anti-mouse IgG secondary antibody. Signals were visualized using ECL detection and exposure of the blots to autoradiographic film. Samples were loaded as follows:

lane 1: protein size marker
lane 2: LC-80 positive control
lane 3: OVCA 433 negative control
lane 4: untreated HEY (control #1)
lane 5: HEY + 2 mM dbcAMP (#1)
lane 6: blank lane
lane 7: untreated HEY (control #2)
lane 8: HEY + 2 mM dbcAMP (#2)


kD
1 2 3 4 5 6 7 8
175
83

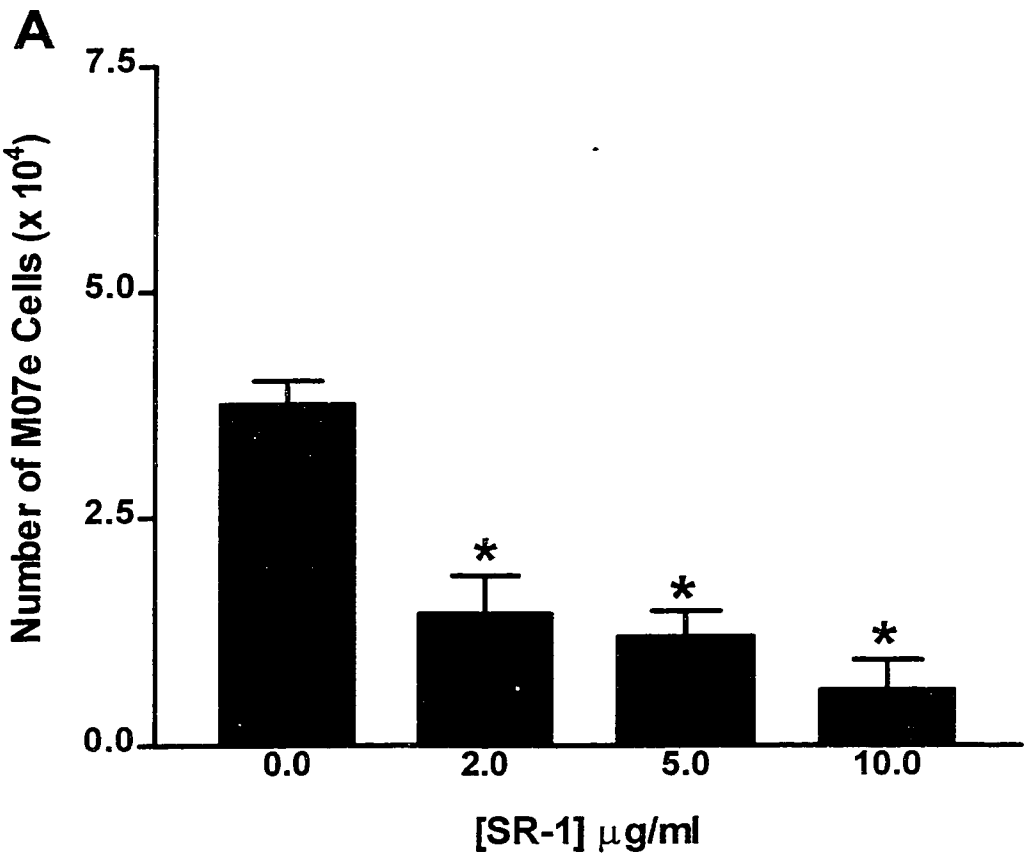
15.5 Effects of Kit activation blockade on dbcAMP-inhibited HEY cells

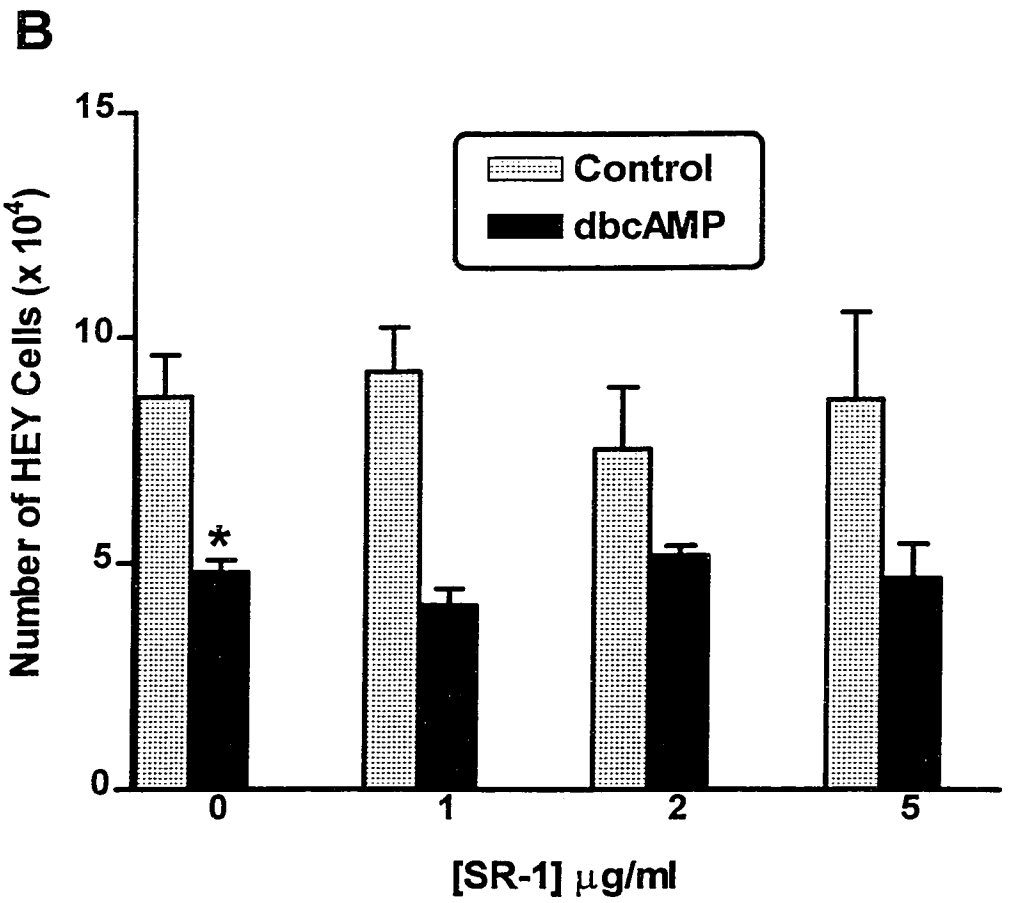
To investigate the role of elevated Kit levels in cAMP-mediated growth inhibition, additional proliferation studies were completed using an anti-Kit neutralizing monoclonal antibody, SR-1. Specifically, this experiment was designed to determine if the growth inhibitory responses mediated by cAMP analogues in HEY cells were directly attributable to the concurrent elevations in Kit protein, *i.e.,* was Kit responsible for cAMP-mediated growth inhibition? For these studies, HEY cells were cultured in the presence or absence of 2 mM dbcAMP, and/or increasing concentrations of SR-1 (0, 1, 2, and 5 μg/ml). HEY cells endogenously express both c-*kit* and KL, and therefore have the potential for autocrine regulation of proliferation following enhanced activation of Kit by KL, which presumably can be blocked by the addition of a neutralizing antibody such as SR-1. Confirmation of the antibody's effectiveness was determined by assessing the growth of the KL-dependent human megakaryoblastic leukemic cell line MO7e, with or without added antibody (Figure 16A). In the presence of exogenous KL (50 μg/ml recombinant human; Immunex, Seattle, WA), MO7e growth is greatly stimulated, however, addition of SR-1 caused prominent inhibition of KL-induced proliferation, at all concentrations tested (61, 68 and 84% inhibition at 2, 5 and 10 μg/ml SR-1, respectively). Figure 16B shows the effects of SR-1 on HEY cell growth both in the presence and absence of dbcAMP. Under basal conditions, culture of HEY cells with increasing concentrations of SR-1 did not affect the rate of proliferation of normal cells. As previously observed, treatment of HEY with 2 mM dbcAMP resulted in a significant decrease (45%) in cell proliferation (control: $8.7 \pm 0.9 \times 10^4$ versus dbcAMP: $4.8 \pm 0.3 \times 10^4$ cells). However, the addition of SR-1 to dbcAMP-inhibited cells did not alter the rate of

proliferation at any dose. Therefore, it would appear that Kit activation is not directly involved in the cascade of events culminating in dbcAMP-mediated inhibition of HEY cell proliferation.

FIGURE 16: ***Effects of blocking Kit activation on dbcAMP-induced inhibition of HEY cell inhibition***

(A) 1.25 x 10^4 MO7e leukemia cells were cultured for 48 h in the presence or absence of 50 μg/ml human KL and/or SR-1 α-Kit monoclonal antibody (0, 2, 5 and 10 μg/ml). Equal numbers o f HEY ovarian carcinoma cells (B) were cultured in the presence or absence of 2 mM dbcAMP and/or SR-1 (0, 1, 2, and 5 μg/ml) for 48 h. Cells were harvested and mean cell number was determined using a hemocytometer. With the exception of (A) M07e + 10 μg/ml SR-1 (n = 1 experiment), each bar represents the number of viable cells from two experiments, and maximum and minimum values are indicated by error bars.


A
7.5
5.0
2.5
0.0
Number of M07e Cells (x 10⁴)
*
*
*
0.0
2.0
5.0
10.0
[SR-1] μg/ml


B
15
10
5
0
Number of HEY Cells (x 10⁴)
Control
dbcAMP
*
0
1
2
5
[SR-1] μg/ml

16. Establishment of Ovarian Tumours in Primary Culture

16.1 Summary of Human Ovarian Tumour Cultures

Since it has been demonstrated that many ovarian tumours express Kit protein (Inoue *et al.*, 1994; A.M. Tonary, personal communication), and that primary cultures may be more representative of cellular growth characteristics *in vivo*, an attempt was made to establish ovarian tumour explants *in vitro*, as a reasonable follow-up in which to examine the regulation of primary tumours in comparison to immortalized ovarian cancer cells. Ultimately, it was hoped that established primary tumours could be maintained for an adequate number of passages in which to complete hormonal, cAMP, and growth factor studies such as had been developed for the HEY and 429 cells, in order to determine the regulation and role of Kit and/or KL in ovarian carcinoma progression.

A brief overview of the primary ovarian tumour culture results are presented in Table 3. Over the course of seven months, a total of 18 fresh tumour samples were received from collaborators at the Ottawa General Hospital (Dr. W. Faught, Dr. M. Fung Kee Fung and Dr. M. Senterman). Of these, a total of 3 tumours from 11 uncontaminated explants of epithelial origin (3/11 = 27%) were maintained in culture for at least three passages, the minimum number of passages necessary to consider the sample as established.

TABLE 3: Brief Overview of Primary Ovarian Tumour Results

Tumour Results	*Number of Tumours*	*Percentage of total received*
Established[a]	**3**	**16.7**
Not established	**8**	**44.4**
Contaminated[b]	**5**	**27.8**
Other[c]	**2**	**11.1**
TOTAL RECEIVED	**18**	**100**

LEGEND:
a = explant cultures maintained at least 3 passages
b = contaminated prior to first passage
c = includes one accidental loss of culture, and one discarded culture (determined to be of stromal, not epithelial origin by pathology report)

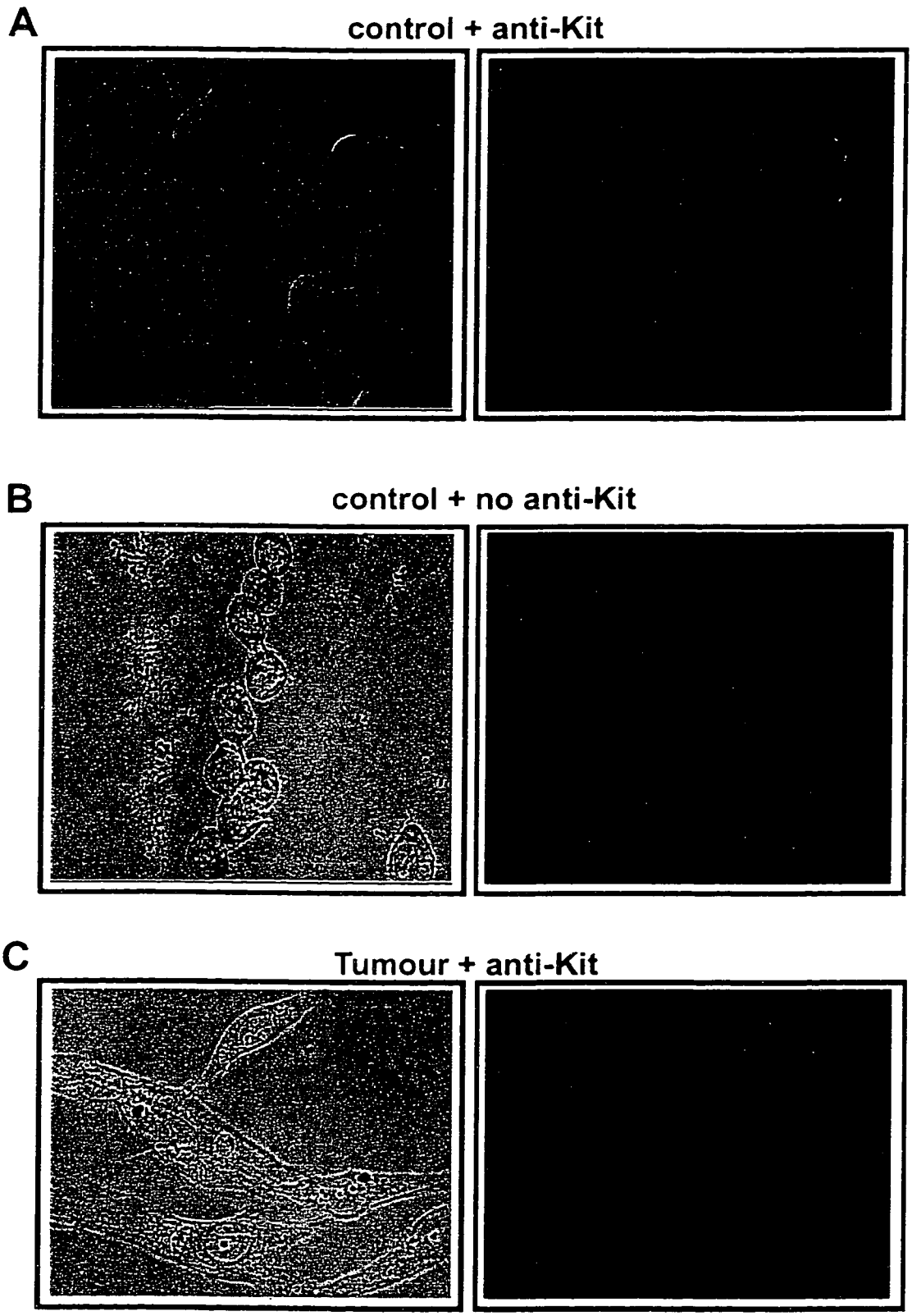
Of the 18 tumours received, five were determined to be contaminated prior to passage 1. Interestingly, the temporal pattern of contamination corresponded to changes in the receiving protocol, such that tumours which were: (1) collected directly into sterile collection media in the operating room instead of being routed through the pathology lab, and (2) digested in a collagenase solution which was filter sterilized prior to use (Nalgene; 0.2 μM syringe filter), were associated with an absence of contamination.

The three successful cultures, identified as N4, N9 and N15 were established under similar conditions. N4 cells were initially collected in OSE media, and following collagenase digestion and sedimentation (~ 8 h), and were divided into OSE and E3 media-containing tissue culture dishes. After four days in culture, a nearly confluent lawn of epithelial-like cells had growth as an adherent monolayer in 35 mm dishes; dishes that were less confluent contained numerous foci of cells. After several passages in culture, N4 cells morphologically appeared to assume more fibroblastic-like and atypical epithelial characteristics, such as long extended processes and irregular cell shapes with voluminous cytoplasm. Determination of Kit and keratin protein expression in N4 cells by Western analysis determined that these primary cells did not express Kit, but did express the epithelial keratin markers (data not shown). N4 cells were the longest growing of the 3 established cultures, and were maintained for almost 10 passages prior to succumbing to bacterial contamination. N9 cells were established under the same media conditions as N4, and by day 10 (d10), these had similar fibroblastic-like morphology as N4 cells. N9 cells were determined to be Kit negative, by immunocytochemistry (Figure 17). N15 cultures were similarly digested and sedimented as for N4 and N9, however, these cells were only cultured in E3 media. N15 cultures appeared

to contain a mixture of predominantly fibroblasts with interspersed epithelial-like foci of tumour cells. The expression of keratin or Kit protein was not determined in these cells.

FIGURE 17: ***Lack of Kit protein expression in N9 ovarian tumours cells as determined by immunocytochemistry***

N9 ovarian tumour cells (p3) were cultured on gelatin-coated coverslips in 35 mm dishes for 24-48 h. A monoclonal anti-Kit antibody (Ab-1) was used to determine Kit protein expression by immunocytochemistry (see **Materials and Methods**, section **11.2**). In (A), photomicrographs depict fluorescent images of the positively expressing LC-80 cells, in which 100% of cells are expressing membrane-bound Kit protein (red fluorescent signal). Negative control for non-specific staining of the antibody was demonstrated by processing duplicate coverslips in the absence of Ab-1 (B). In (C), coverslips containing N9 cells were incubated in the presence of Ab-1. There is no detectable evidence of Kit protein expression in these cells.


DIC
Fluorescence
A
control + anti-Kit
B
control + no anti-Kit
C
Tumour + anti-Kit

DISCUSSION

The purpose of the present study was to test the hypothesis that the c-*kit*/KL system is involved in ovarian cancer cell proliferation in response to factors which are produced by, and/or exert effects on, the ovary. Two ovarian cancer cell lines, HEY and OVCA 429, were identified which express both c-*kit* and KL mRNA, and were used as a model system for determining hormonal and growth factor regulation of the c-*kit*/KL growth factor system in ovarian cancer cells. Particular interest was directed towards associating changes in cellular proliferation with alterations in the abundance of either transcript, as a means of investigating the potential autocrine regulation of growth in these two cell lines. The experiments reported here demonstrate that under various conditions, the exogenous addition of steroid and gonadotropic hormones or growth factors did not alter either the proliferative capacity or the transcript expression of c-*kit* or KL in both HEY and OVCA 429 cells. However, c-*kit* mRNA and protein expression in the HEY ovarian carcinoma cell line was significantly upregulated by membrane-permeable analogues of cAMP, and this was associated, but not functionally linked with growth inhibition *in vitro*. This is the first demonstration of exogenously induced changes in Kit expression in an ovarian carcinoma cell line.

Using a variety of conditions, neither HEY cell growth, nor expression of c-*kit* or KL transcripts, were altered in response to stimulation by EGF, TGF-α or TGF-β. One potential explanation for the lack of response induced by TGF-α and EGF in both 10% and 1% FCS-containing cultures is that these and other growth factors might be present in sufficient quantities in the provided FCS in which to mask the effects of the added growth factors. Fetal calf serum is a biochemically *undefined* additive, which is believed to contain numerous

factors (growth factors, hormones, nutrient binding carrier proteins, immunoglobulins, vitamins, minerals and ions), which interact to support and enhance the attachment, growth and differentiation of cultured cells. In these studies, initial experiments completed in 10% serum demonstrated no observable differences in HEY cell proliferation in response to any of the added growth factors, and repeat experiments performed with cells cultured in 1% serum, produced a similar lack of response in growth factor-treated HEY cells. Most likely, the nutrient environment of even 1% serum-containing media was sufficient to adequately stimulate the proliferation of HEY cells, such that these cells were not responsive to addition of surplus growth factors. This finding is in contrast to that of Jindal *et al.* (1994) who reported that HEY cells were mildly growth stimulated by TGF-α and EGF, as assessed by a 2-fold increase in [^{3}H]-thymidine incorporation. The key difference between the experiments presented here, and those of Jindal *et al.*,is that all of our experiments were carried out in media containing variable amounts of serum (1% -10%), while those of Jindal *et al.* were completed using cells that had reached a quiescent state after 48 h in serum-depleted media. As a means of confirming Jindal's studies, those experiments were repeated exactly as reported, but in various attempts, HEY cells would not survive in serum-free media for longer than 24 h, even in the presence of added supplements such as ITS, BSA or glutamine, and no effects on proliferation were observed with the addition of EGF, TGF-α or TGF-β. The most likely explanation for this contradictory response is the inherent characteristic of some cell lines to undergo phenotypic and functional changes with increasing numbers of passages away from the original tissue source. The HEY cell line was originally established 13 years ago and the stocks used by both Jindal *et al.* and our own lab were

obtained from separate secondary sources. One could propose that the HEY cells maintained within our laboratory are far enough removed from the population of cells which Jindal *et al.* used that they no longer have similar responses to some external stimuli.

The predominant effect of TGF-β on most normal cells including OSE *in vitro* is inhibition of proliferation (Nilsen-Hamilton, 1990). The lack of growth inhibitory response produced by addition of TGF-β to our HEY cell cultures is in agreement with other investigators who have shown that transformed cell lines of various cell types are usually resistant to growth inhibition by TGF-β, compared to their normal non-transformed counterparts (Barnard *et al.*, 1990; Huggett *et al.*, 1990; Reiss *et al.*, 1993; Valverius *et al.*, 1989). Refractoriness to TGF-β has been shown for advanced breast epithelial tumours (*in vivo*; Reiss & Barcellos-Hoff, 1997), and for several ovarian carcinoma cell lines as well (Berchuck *et al.*, 1990b; Khan *et al.*, 1997), and it has been suggested that this response may allow some cancers to attain a more malignant phenotype following escape from the growth inhibitory actions of TGF-β. The resistance to TGF-β *in vitro* does not appear to be correlated with loss of receptor expression, as some resistant cell lines exhibit similar binding affinities and receptor numbers, and show a comparable pattern of stimulated expression of secreted proteins as normal cells, in response to added TGF-β. (Huggett *et al.*, 1990). With regard to the situation *in vivo*, it has therefore been suggested that the compromise of growth-regulatory pathways in some cancer cells may allow these tumours to escape normal growth inhibitory pathways such as that mediated by TGF-β.

In another series of experiments, HEY and OVCA 429 cell line responses to steroid hormone stimulation were assessed by cell counting and examination of c-*kit* and KL mRNA

expression. Under the conditions used in these experiments (48 h cultures, 10% serum-containing media), exogenous addition of E2, P4, or T did not alter cell proliferation of either cell line, as assessed by final cell counts using a hemocytometer. While there were tendencies for a number of the treatments to seemingly induce either growth inhibitory or growth stimulatory effects, these differences in cell proliferation were not statistically significant and probably reflect only inter-experimental variations. Substantial evidence has accumulated which would suggest a role for steroid hormones, and particularly E_2 on ovarian carcinoma cell proliferation (Langdon *et al.*, 1994, Nash *et al.*, 1989; Wimalasena *et al.*, 1991), and we hypothesized that the effects of agonists on HEY or OVCA 429 cell proliferation would be associated with altered expression of c-*kit* or KL. Several possibilities arise to explain the absence of proliferative responses observed in these cells treated with exogenous hormones, not the least of which is that these cell lines did not respond because they may not express the appropriate receptors. At the time of these experiments, the steroid-receptor status of the HEY and OVCA 429 cells was not known. Therefore, due to the lack of response of these cells in 10% serum-containing media, it became necessary to determine if perhaps the absence of steroid-induced effects on proliferation rates were due, at least in part, to the presence of steroids within the serum in which the cells were cultured, which were potentially masking the effects of added steroid hormones. Another ovarian carcinoma cell line, OVCA 433, which is known to possess functional estrogen receptors (Scambia *et al.*, 1990) was used as a positive control for the stimulatory effects of exogenously added E_2 (500 nM) in media containing charcoal-treated serum (for steroid-removal). While added E_2 caused significant growth stimulation of OVCA 433 cells (as expected), the proliferation

of OVCA 429 cells was still not altered, even under these modified conditions (charcoal-treated serum), or with 10-fold greater E_2 (5000 nM).

The lack of growth responsiveness of OVCA 429 cells by estradiol has been shown for different ovarian carcinoma cell lines by other investigators. For example, Langdon *et al.*, (1994) reported that only 3 out of 8 ovarian carcinoma cell lines responded with increased proliferation to 17β-estradiol stimulation. While these responding cell lines displayed moderate levels of estrogen receptor as determined by protein analysis, other cell lines which strongly express estrogen receptors have displayed contrasting proliferation responses to estradiol. *In vitro*, SKOV3 ovarian carcinoma cells are resistant to E_2 stimulation of proliferation (Hua *et al.*, 1995), while PE01 and PE04 cells are responsive (Crew *et al.*, 1992; Langdon *et al.*, 1993; Simpson *et al.*, 1998). However, when PE04 cells are xenografted into nude mice, exogenous E_2 causes suppression of proliferation in these cells (Langdon *et al.*, 1993). Taken together, these findings would suggest that, in receptor-bearing cell lines, not all ovarian carcinoma cell lines respond equally to stimulation with E_2. Whether these contrasting responses are representative of inherent differences in ovarian tumour characteristics *in vivo*, or rather, if it reflects the ability of various environments to influence cellular activity by exogenous factors, remains to be determined.

Recently, the estrogen receptor status of HEY cells was investigated using RT-PCR, and the lack of detectable transcripts for either of the estrogen receptor forms (α or β) was reported for these cells (Brandenburger *et al.*, 1998). Therefore, it is clear that the inability of E2 to promote changes in the proliferation or gene expression of HEY cells is due to the absence of ligand-binding estrogen receptors. Although the expression of P_4 and T receptors

in HEY cells (and OVCA 429) has not been determined, this could also provide explanation for the similar lack of effects mediated by either P_4 or T on HEY cell proliferation.

Another contending possibility for the absence of steroid and gonadotropic regulation of proliferation of HEY and OVCA 429 cells, is that these particular cell lines are not responsive to exogenous hormonal stimulation at all, or instead, they were responsive in parameters which were not measured in this study. For example, SKOV3 ovarian carcinoma cells, which express functional estrogen receptors, have been demonstrated to respond to exogenous E_2 by stimulating the expression of the early growth response genes, c-*myc* and c-*fos*, however, their proliferation is not altered in response to E_2 or to antiestrogens (Hua *et al.*, 1995). Furthermore, another possible argument for the results presented herein could be that the hormone concentrations used in these experiments were not high enough to regulate proliferation and/or gene expression. This is not very likely, as the doses used in these studies were equivalent to, or greater than, those reported as being effective in the literature (FSH 100-300 ng/ml, 10-100 ng/ml hCG: Laitinen *et al.*, 1995; E_2 (0.1 nM and 10 nM): Langdon *et al.*, 1990; E_2 (1 pM to 1 μM): Langdon *et al.*, 1993; 100 mIU/ml hLH: Kurbacher *et al.*, 1995). Continuation of these studies under more extensive culture conditions (including dose- and time-response studies using a number of concentrations and timepoints) would effectively address this criticism.

Gonadotropic hormones have been suggested to assume an integral role in the transformation of OSE cells to a malignant phenotype, as a result of increased circulating concentrations during the (peri)menopausal period. Since both gonadotropins, and particularly FSH, are elevated at the time of menopause (Reyes *et al.*, 1977), and receptors

for FSH and LH/hCG have been demonstrated in both normal OSE cells and transformed ovarian carcinoma cells (Kammerman *et al.*, 1981), the hypothesis of this thesis postulated that exogenous FSH and/or hCG would stimulate HEY and OVCA 429 cell proliferation, and that this effect might be related to upregulation of c-*kit* and/or KL. However, culture of the c-*kit*/KL expressing cell lines, HEY and OVCA 429, with FSH or hCG did not alter the proliferation or gene expression of these ovarian cancer cell lines. Preliminary analyses have determined that HEY cells express one of the minor transcripts for the FSH receptor (FSH-R), while both HEY and OVCA 429 cells express transcripts for the LH-R, but it has not yet been determined if any of these transcripts encode for functional protein (A.M. Tonary, personal communication). In culture, primary granulosa cells, which are the primary gonadotropin receptor-bearing cells, routinely lose expression of gonadotropin receptors during immortalization (Amsterdam *et al.*, 1979). Therefore, the lack of regulation of proliferation by exogenously added gonadotropic hormones in these cells due to receptor loss, is not an unlikely possibility, and remains to be determined.

As a means of pursuing the apparent lack of effect of gonadotropic hormones, HEY cells were cultured in the presence of 2 membrane-permeable analogues of cAMP, and an adenylate cyclase activator, each of which acts to elevate intracellular levels of cAMP. In the presence of these molecules, the downstream signalling pathway of both gonadotropin receptors were presumably stimulated in the absence of external ligand-receptor binding. Addition of dbcAMP, 8-bromo-cAMP or cholera toxin induced significant and dose-dependent *inhibition* of HEY cell proliferation over a 48 h culture period. This finding was in direct contrast to the expected result, which had proposed that since gonadotropins are key

stimulators of ovarian carcinoma cell proliferation, cAMP-treated HEY cells would be induced to proliferate. While it cannot be ruled out that similar effects *in vivo* may be attributable to molecules other than the gonadotropins (such as growth factors, prostaglandins, acetylcholine and serotonin) which likewise signal through a cAMP intermediate, an equally likely possibility remains that gonadotropic hormones, and particularly FSH, are potent regulators of ovarian cancer cell proliferation, *in vivo,* via cAMP signalling. However, the presentation of cAMP, an effector for gonadotropic signalling, at conceivably greater levels than those presented physiologically by the gonadotropins themselves, would argue that the responses obtained herein may not be truly representative of those observed *in vivo*. The growth inhibitory effects mediated by cAMP analogues have been well-documented for other types of cell lines, including those of tumour origin (meningioma, Huttner *et al.*, 1996; A431 epidermoid carcinoma: Kamiya *et al.*, 1995; U-937 lymphoma, Laskin *et al.*, 1990; TE85 osteosarcoma, Mohan *et al.*, 1991; pancreatic carcinoma, Ohmura *et al.*, 1993), however, this is the first demonstration of cAMP-induced suppression of proliferation in an ovarian cancer cell line. DbcAMP was also growth inhibitory to OVCA 429 cells, suggesting the potential generalization of this cAMP-inhibitory effect among ovarian carcinoma cell lines.

An unexpected finding in this project was the observation that inhibition of HEY cell proliferation by these membrane permeable cAMP analogues was associated with marked *increases* in c-*kit* mRNA and protein expression. This was in marked contrast to the predictions of our hypothesis, which had postulated that upregulation of c-*kit* would be associated with growth stimulation, and not growth suppression. Cyclic AMP-mediated

upregulation of c-*kit* has been shown in K562YO human erythroleukemia cells (Ogawa *et al.*, 1995) and in differentiated F9 mouse teratocarcinoma cells (Nishina *et al.*, 1992), however, this finding has never been reported for ovarian carcinoma cells. Furthermore, concentrations which had produced the greatest growth inhibition, were found to induce the greatest upregulation of c-*kit* mRNA transcripts. This finding was surprising, as it had been hypothesized that stimulation of Kit expression and activation in ovarian carcinoma cells would drive greater proliferation, and not suppression, of cells with enhanced levels of c-*kit*. At least three arguments may be proposed to explain this apparent discrepancy with our expected results. A series of related papers by Natali et al. (1992b; 1992c; 1995) revealed that some tumours (breast, melanoma and thyroid) progressively lose expression of the Kit receptor with enhanced growth and invasion. Furthermore, transfection of c-*kit* into a highly metastatic c-*kit*-negative human melanoma cell line, triggered apoptosis and suppressed their tumourigenic potential upon exposure to KL (Huang *et al.*, 1996). It was suggested that loss of Kit receptor expression in some tumours may allow these cells to evade the potential autocrine induction of programmed cell death by endogenous ligand. Therefore, in light of the results presented in this work, it is possible that similar events are occurring here. Enhanced c-*kit* expression in a cell line which co-expresses KL was associated with inhibition of ovarian carcinoma cell proliferation, and may suggest that ovarian carcinomas are also responsive to KL-mediated apoptosis as a means of controlling metastatic growth.

The second explanation for the observed results may be that upregulation of c-*kit* expression occurred as a consequence of the substantial inhibition of proliferation following exposure of HEY cells to cAMP analogues. Using *in vitro* assay systems, KL-activated Kit

has been demonstrated as a key survival mechanism for migratory primordial germ cells (Dolci *et al.*, 1991; Godin *et al.*, 1991), immature melanoblasts (Mackenzie *et al.*, 1997) and type A spermatogonia (Packer *et al.*, 1995). Although no upregulation of KL was detected in cAMP-inhibited cells, upregulation of c-*kit* may be the initial step in a series of events intended to ultimately promote the survival of these growth-suppressed cells.

A third possibility which deserves serious consideration is that the dual responses induced by cAMP (c-*kit* upregulation and concurrent inhibition of HEY cell proliferation) are temporally related, but not functionally linked. While the complete downstream signalling pathway of c-*kit* is not fully understood, c-*kit* has been associated with responses that are *not* direct actions on cell proliferation in some cell types (eg. differentiation of murine erythroleukemia cells, Hino *et al.*, 1995). In order to assess the association between c-*kit* and cell proliferation changes in the presence of cAMP, preliminary experiments were completed using an anti-Kit neutralizing antibody in cultures of dbcAMP-treated HEY cells. This set of experiments demonstrated that dbcAMP-mediated inhibition of HEY cell proliferation was *not* reversed by blocking the activation of Kit by KL, indicating that in this cell system, Kit is not directly involved in the promotion of cAMP-induced effects on cell proliferation. This would argue more favourably for the survival hypothesis proposed above, and lends less support to the possibility of enhanced c-*kit* expression mediating proliferation suppression in ovarian cancer, as has been otherwise described for melanoma, breast, and thyroid tumours.

A final aspect of this project included an attempt to establish primary human ovarian tumour cultures, in order to compare the relative effects of the aforementioned ovarian

factors on both continuous cell lines and primary cell cultures. Specifically, we had hoped to establish and maintain primary cultures of epithelial ovarian tumours which expressed abundant levels of Kit protein. Of 11 clinical tumour samples obtained which met our basic criteria (epithelial origin, non-contaminated), 3 ovarian tumour explants were established in culture for a minimum of three passages, and 2 of these were determined not to express the Kit receptor. Despite varied success in the literature, average success in the frequency of established ovarian cancer cells are approximately 50% (Lounis *et al.*, 1994), and, furthermore, most investigators report only the established primary cultures and/or cell lines which they have produced, and not the total number of attempts made. Therefore, the results presented here are in keeping with the generally accepted belief that primary cultures are notoriously difficult to establish, and even more difficult to maintain for long-term growth. However, while this attempt was curtailed, tremendous benefit could be gained from such a system by those interested in pursuing the involvement of the c-*kit* protooncogene in ovarian tumourigenesis.

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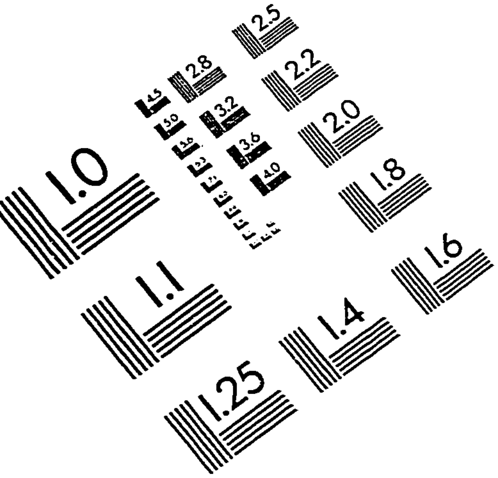
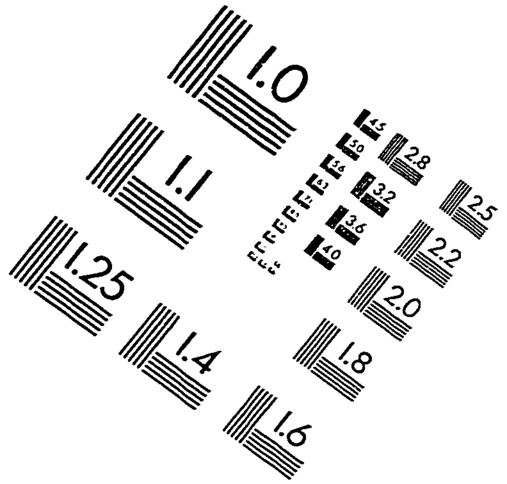
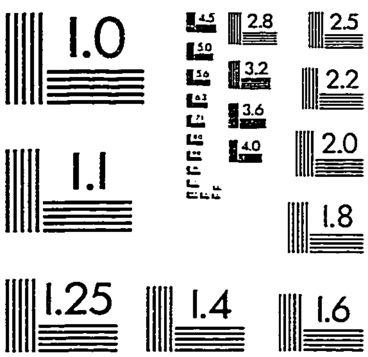
IMAGE EVALUATION TEST TARGET (QA-3)







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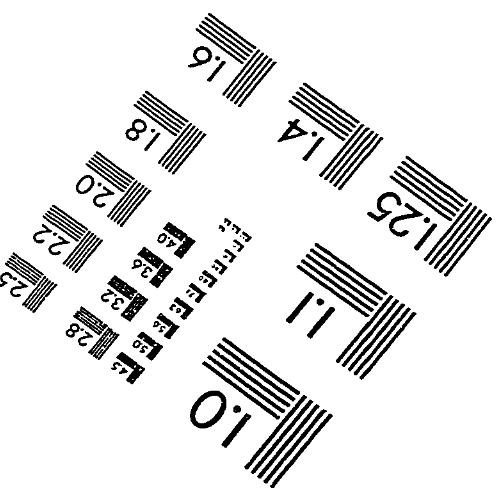

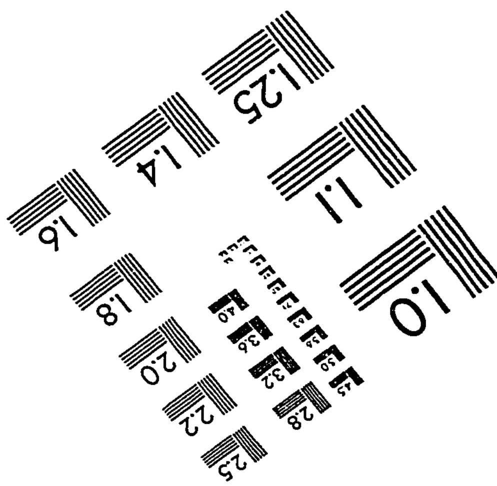

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