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THE EFFECTS OF STROMAL-DERIVED FACTORS AND GONADOTROPINS ON RAT OVARIAN SURFACE EPITHELIAL CELL PROLIFERATION AND EXPRESSION OF C-KIT AND KIT LIGAND

by

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DEDICATION

A mes deux amours, sans eux je n'aurais pas fait cette maîtrise.

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ABSTRACT

Although ovarian cancer is rare, it is the most deadly of gynaecological cancers. Unfortunately, still very little is known about the cells that give rise to 90% of ovarian cancers, the ovarian surface epithelial (OSE) cells, and much of the available data remains controversial. This project was designed to address the possible involvement of ovarian stromal/thecal cells in the regulation of OSE cell growth and Kit and KL expression. Such interactions are probably involved in normal OSE-stromal/thecal cell activities as well as in interactions occurring within inclusion cysts and leading to ovarian tumour formation.

The regulation of rat OSE (ROSE) cell growth by theca-derived factors and gonadotropins was investigated by proliferation experiments and cell counts. The modulation of Kit and Kit ligand (KL) messenger ribonucleic acid (mRNA) expression in these cells by the same factors was investigated by Northern blot analysis and reverse transcription-polymerase chain reaction (RT-PCR).

Transforming growth factor-beta (TGF-β) was found to inhibit the growth of both ROSE 199 and primary ROSE cells. Transforming growth factor- alpha (TGF-α)and epidermal growth factor (EGF) on the other hand stimulated the growth of primary ROSE but not immortalized ROSE cells. While human chorionic gonadotropin (hCG) also stimulated the proliferation of primary ROSE cells, follicle stimulating hormone (FSH) had no effect on the growth of these cells. Stromal-conditioned medium (SCM) also had a growth inhibitory effect on ROSE cells, but adding TGF-β neutralizing antibody did not eliminate this effect. All three exogenous growth factors also decreased KL mRNA

expression but none of the factors investigated were found to induce expression of Kit in the primary ROSE cells by Northern blot.

The findings that stromal/thecal-derived factors and hCG can regulate growth and KL expression in OSE cells suggests that the paracrine interactions between these two cell types may involve a cascade of growth factors produced by either cell type and be regulated during the ovulatory cycle. Interactions gone awry may lead to the transformation of OSE cells and ovarian tumour formation.

LIST OF FIGURES

LIST OF ABBREVIATIONS

Ab	antibody
ANOVA	analysis of variance
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
DES	diethylstilbestrol
DEPC	diethylpyrocarbonate
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGF-R	epidermal growth factor receptor
FBS	fetal bovine serum
FCS	fetal calf serum
FITC	fluorescein
FSH	follicule stimulating hormone
FSH-R	follicule stimulating hormone receptor
H-199B	HEPES-buffered Medium 199 with BSA
HBSS	Hank's Balanced Salt Solution
hCG	human chorionic gonadotropin
HGF	hepatocyte growth factor
hOSE	human ovarian surface epithelium

IgG	immunoglobulin G
kD	kilodalton
KGF	keratinocyte growth factor
KL	Kit ligand
KOAC	potassium acetate
LH	luteinizing hormone
LH-R	luteinizing hormone receptor
MEM	minimal essential medium
mRNA	messenger ribonucleic acid
MW	molecular weight
OSE	ovarian surface epithelium
PBS	phosphate buffered saline
rpm	rotations per minute
ROSE	rat ovarian surface epithelial
RT-PCR	reverse transcription-polymerase chain reaction
SCM	stromal-conditioned media
SDS	sodium dodecyl sulfate
S.E.M.	standard error of the mean
SSC	saline sodium citrate
Sl	Steel locus
S-PBS	Stockholm's phosphate buffered saline
TGF-α	transforming growth factor-alpha
TGF-β	transforming growth factor-beta

TGF-β-RI/RII	transforming growth factor-beta receptor I and II
UV	ultraviolet light
V	volts
W	white spotting locus
w/v	weight/volume

INTRODUCTION

1. Ovarian Cancer

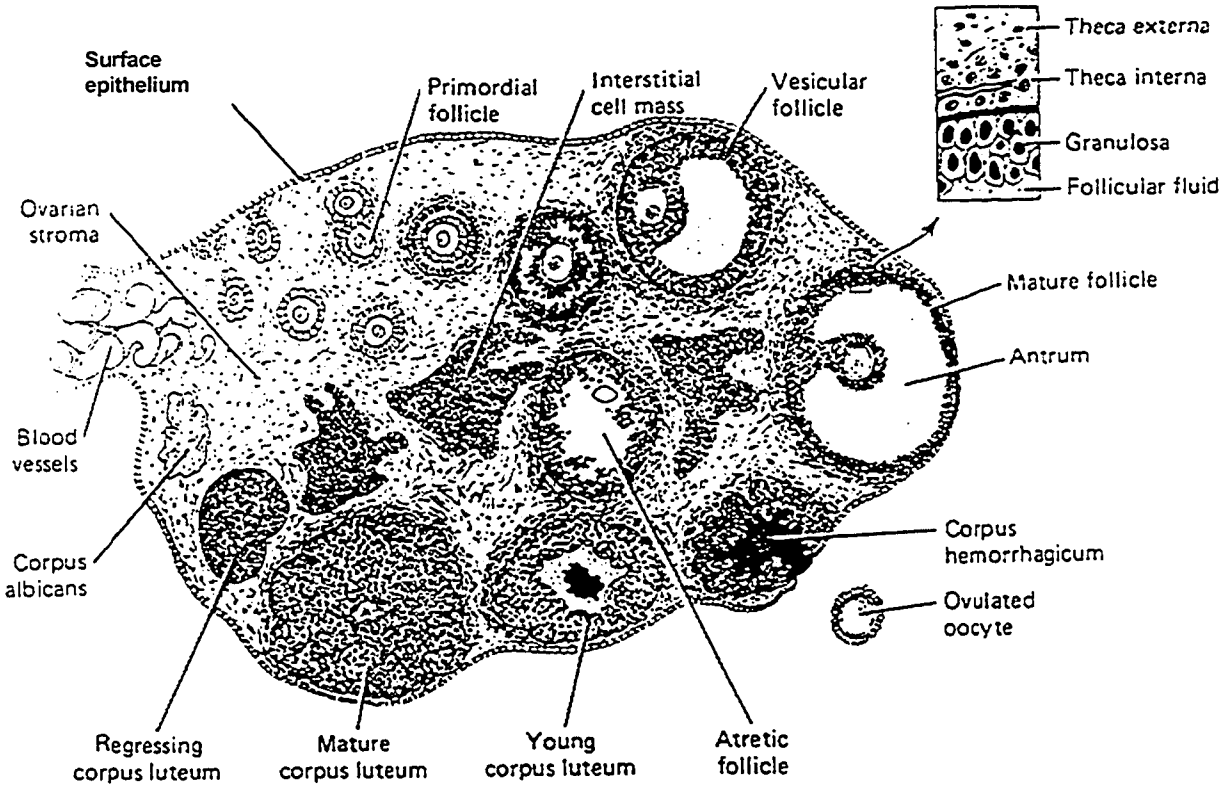
1.1 Basic Physiology of the Ovary

The ovary is a multilayered organ in which different cell types communicate in order to coordinate and regulate follicular growth and ovulation. As depicted in Figure 1, the pre-menopausal ovary is comprised of many follicles at various stages of development, each containing a single oocyte surrounded by layers of granulosa cells which increase in number as the follicle matures. The thecal cells, which are derived from differentiated stromal cells (Hirshfield, 1991), surround the granulosa cells in the more mature follicles and provide structural integrity. A number of corpora lutea are also present but the undifferentiated interfollicular stroma make up the bulk of the ovary. Finally, the surface epithelial cells surround the ovary as a single layer of cells varying from squamous to columnar. These epithelial cells are mesodermal in origin, embryologically derived from mesenchymal tissue (reviewed in Murdoch, 1996), but the adult OSE is relatively uncommitted and closer to a stem cell form than other coelomic epithelial derivatives. A basal membrane overlying a fibrous tunica albugenia supports this single continuous layer of epithelial cells. The OSE cells are held together laterally by desmosomes and gap and tight junctions (reviewed in Murdoch, 1996).

At the time of ovulation, OSE cells secrete lysosomal proteases that can apparently degrade the tunica albugenia and underlying extracellular matrix and thecal cells (Bjersing and Cajander, 1974). Those OSE cells directly apposed to the impeding

Figure 1: Ovary

The ovarian tissue is made up of many different cell types including epithelial, stromal/thecal, granulosa, and oocyte. Here the major cell types are depicted at various stages of differentiation during the ovarian cycle. Adapted from Ganong, (1981).


Surface epithelium
Primordial follicle
Interstitial cell mass
Vesicular follicle
Theca externa
Theca interna
Granulosa
Follicular fluid
Ovarian stroma
Mature follicle
Antrum
Blood vessels
Corpus albicans
Corpus hemorrhagicum
Ovulated oocyte
Regressing corpus luteum
Mature corpus luteum
Young corpus luteum
Atretic follicle

site of rupture undergo apoptotic cell death and are shed from the ovarian surface (Murdoch, 1994; Murdoch, 1995). After ovulation, the OSE cells undergo a burst of proliferation to repair the ovulatory wound and restore the continuity of the epithelium (Osterholzer *et al.*, 1985a). Other than its role in ovulation, little is known about the normal function of OSE and factors that affect cell activity.

1.2 Epidemiology of Ovarian Cancer

Ovarian cancer is the fifth most frequent cancer in women behind lung, breast, colorectal, and pancreas, the fourth leading cause of cancer-related deaths in Canada and causes more deaths than all other gynaecologic cancers combined (National Cancer Institute of Canada, 2000). Approximately 90% of ovarian cancers arise from the single continuous layer OSE cells that comprise the external surface of the ovary (Bell, 1991).

1.3 Diagnosis and Screening of Ovarian Cancer

Depending on the clinical advancement of the disease, ovarian cancer is diagnosed as one of four basic stages: stage I is defined by growth limited to one ovary; the cancer is defined as stage II when growth involves one or both ovaries with pelvic extension; stage III is characterized by the development of tumours involving one or both ovaries with peritoneal implants outside the pelvis; and stage IV is defined by ovarian growth with distant disseminated metastases (Skarin, 1997). Unfortunately, most ovarian cancer patients are diagnosed with advanced stage disease since they are asymptomatic until the disease has metastasized, thereby compromising the patient's survival and making it the most fatal of all gynaecologic cancers. In such advanced stage ovarian cancers, patients have a 5-year survival rate of less than 20% (Holschneider and Berek, 2000).

The most common ovarian tumours are classified based on cellular and structural features reminiscent of normal adult tissues of Müllerian origin, which is not surprising since the OSE is embryologically related to the Müllerian system. The *serous* tumours are similar in appearance to the epithelium of the fallopian tube, the *mucinous* tumours that of the endocervix and the *endometrioid* tumours to that of the endometrium. Although less common, *clear cell* tumours comprise the most lethal subtype of ovarian cancer (Bell, 1991). It is unknown whether the molecular etiology between these tumour subcategories is distinct (Testa *et al.*, 1994).

Due to the limitations in currently available methods of detection and to the low incidence of this malignancy, screening for early detection of ovarian cancer has been restricted to women determined to be at increased genetic risk for ovarian cancer. Prophylactic oophorectomy is also often recommended to women with predisposition to the disease. One screening method relies on the determination of serum tumour markers such as CA-125 for the detection of ovarian cancer. Auersperg *et al.* (1995) have reported that human ovarian surface epithelial (hOSE) cells from cancer prone women are more stable with regard to CA-125 production, compared to normal risk women. The epithelial marker CA-125 disappeared within a few passages in cultured ovarian epithelial cells from women with no family history of ovarian cancer, in contrast with the cells from women with a family history whose cells continued to produce CA-125 in late passages. This group also showed that the OSE cells in inclusion cysts were more consistently CA-125 positive (Auersperg *et al.*, 1995). Unfortunately, this screening method has proved too insensitive and nonspecific to be used as a single test. More sensitive but more expensive techniques of pelvic ultrasound screening, including abdominal and

transvaginal sonography have been more successful at identifying asymptomatic women with stage I lesions. Second-look laparotomy is sometimes used to assess disease status after therapy (Murdoch, 1996; Lynch *et al.*, 1998).

1.4 Treatment of Ovarian Cancer

Current standard treatment for advanced-stage ovarian cancer includes cytoreductive surgery followed by combination chemotherapy with paclitaxel and a platinum drug (eg, cisplatin, carboplatin). Although high response rates are often obtained with this regimen, the majority of initially responsive patients become resistant to most if not all drugs to which they are exposed (Auersperg *et al.*, 1998). Multidrug resistance hence is a major clinical problem in ovarian cancer. New treatment strategies must therefore be developed as resistance mechanisms (altered drug transport, increased drug inactivation, altered drug target, increased drug sequestration, and increased deoxyribonucleic acid (DNA) repair activity and drug damage tolerance) are being uncovered.

1.5 Oncogenes in Ovarian Cancer

Both oncogenes and tumour suppressor genes have been implicated in cancer. Oncogenes normally encode proteins that are growth stimulatory. They are considered dominant transforming genes since alterations leading to their activation, such as mutations, deletions, overexpression or translocations, of a single allele can lead to the transformation of cells. Tumour suppressor genes, on the other hand, encode proteins that normally inhibit cell proliferation. Thus inactivation of both alleles is necessary to

transform cells. Tumour-suppressor genes are therefore recessive transforming genes (Berchuck *et al.*, 1992a).

Studies on oncogenes, tumour suppressors and mutator genes with respect to ovarian cancer have been extensively reviewed (Berchuck *et al.*, 1992a; Auersperg *et al.*, 1998; Aunoble *et al.*, 2000). The mutations and/or overexpression of a few proto-oncogenes, including HER-2/*neu* (c-*erb*B-2), K-*ras*, cFMS, c-*myc* and AKT2, have been implicated in the pathogenesis of sporadic ovarian cancer. Tumour suppressor genes implicated in ovarian cancer include p53, *BRCA1* and *BRCA2*. The involvement of these oncogenes and tumour suppressor genes concerning patient survival, response to chemotherapeutic drugs and tumour grade/histology is an active area of investigation.

1.6 Etiology of Ovarian Cancer

1.6.1. The Incessant ovulation hypothesis

Several theories, which are by no means mutually exclusive, have been put forward regarding the etiology of epithelial ovarian cancer. Fathalla's "incessant ovulation" hypothesis is one such theory (Fathalla, 1971). This hypothesis states that the repeated rupture of the OSE cell layer followed by rapid proliferation as occurs with repetitious ovulation provides opportunity for mutation and malignant transformation of OSE cells. Because cells of the OSE replenish themselves through the division of generative rather than replenishment stem cells, accumulated mutations would be passed on exponentially during each ovulatory event, increasing the chance that the next generation of cells may carry the malignant phenotype (Godwin *et al.*, 1992).

In support of the idea that increased (and repeated) cell division at the time of ovulation may lead to increased DNA damage, is a molecular epidemiologic study suggesting that women who have more total ovulatory cycles in their life time are at higher risk of having tumours overexpressing mutant p53 if they develop ovarian cancer (Schildkraut *et al.*, 1997).

The incessant ovulation hypothesis is further supported by both experimental and epidemiological data. Godwin *et al.* (1992) and Testa *et al.* (1994) have reported the spontaneous transformation of rat ovarian surface epithelial (ROSE) cells subjected to repeat subculturing in vitro for more than 20 passages, as would occur during repeated ovulation and repair. Many of the repeatedly subcultured cell preparations had acquired features associated with transformation such as the loss of contact inhibition, the capacity for substrate-independent growth, tumorigenicity and cytogenic abnormalities (Godwin *et al.*, 1992; Testa *et al.*, 1994).

Epidemiological studies indicate that events that decrease the frequency of ovulation, such as oral contraceptives (Gross and Schlesselman, 1994; Vessey and Painter, 1995), lactation (Risch *et al.*, 1994) and multiparity (Adami *et al.*, 1994; Hankinson *et al.*, 1995; Hartge *et al.*, 1994) also reduce the risk of ovarian cancer. On the other hand events that increase the frequency of ovulation such as nulliparity and, the use of fertility drugs tend to increase the risk of developing ovarian cancer (Rossing and Weiss, 1995; Franceschi *et al.*, 1991; Negri *et al.*, 1991; Harris *et al.*, 1992; Whittemore *et al.*, 1992).

In accord with the above epidemiological data is the observation that common epithelial tumours of the ovaries rarely occur in animals other than humans. This may be

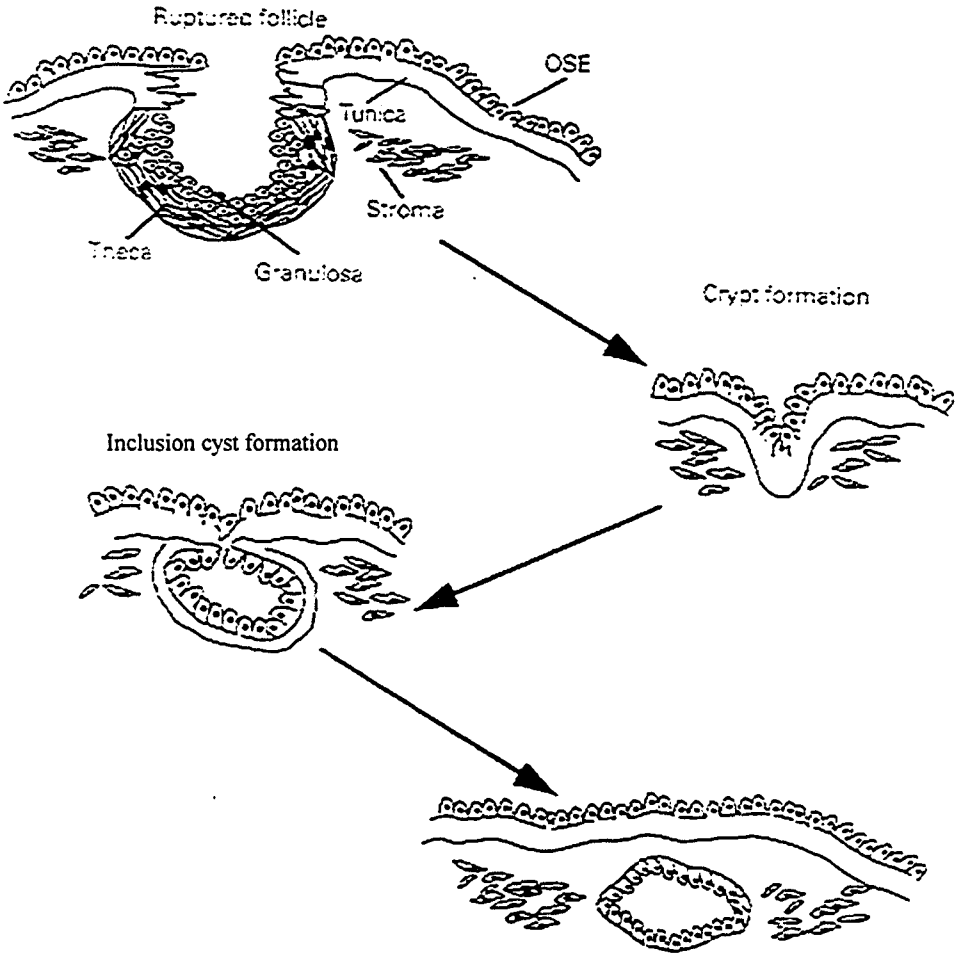
explained by the fact that females of most animal species are normally pregnant or lactating for the bulk of their adult lives thereby ovulating unfrequently, while nulliparity and thus repetitious ovulation is peculiar to the human female of present-day society. The only exception to the infrequent ovulation seen in animals, but which also supports Fathalla's hypothesis, is the report of high incidence of adenocarcinoma of the ovary in domestic laying hens forced to incessantly produce eggs for 2 years without seasonal rest periods (Fredrickson, 1987). Since cells of the OSE do not normally undergo the wound-repair cycle more than once during the breeding season of an animal, it has been suggested that the OSE has not been selected by evolution for the capacity to be wounded and repaired repeatedly, as have cells of the skin or gastrointestinal tract (Godwin *et al.*, 1992).

1.6.2. The formation of inclusion cysts

The process of ovulation per se and not necessarily repeated ovulation has also been suggested to be involved in the development of ovarian cancer. As depicted in Figure 2, the post-ovulatory healing process can lead to the formation of crypts in the ovarian surface. These crypts can become internalized within the ovarian stroma, where they form inclusion cysts lined with OSE cells. Cramer and Welch (1983) have proposed that these inclusion cysts are the sites of origin of ovarian tumours (Cramer and Welch, 1983). Receptors to many hormones and growth factors produced by the ovary are present on OSE cells. Although these factors may not normally be found in the peritoneal cavity where OSE cells reside, the formation of inclusion cysts which entrap the OSE cells within the ovarian cortex may expose them to factors to which they are not normally

Figure 2: Formation of inclusion cysts

Healing of the ovulatory wound creates a crypt in the ovarian surface, which can become internalized into the ovarian stroma where inclusion cysts lined with OSE cells may be formed. These inclusion cysts are thought to be the sites of origin of many ovarian tumours. Adapted from Ghahremani *et al.* (1999).


Ruptured follicle
OSE
Tunica
Stroma
Theca
Granulosa
Crypt formation
Inclusion cyst formation
OSE-lined inclusion cyst

exposed, or to concentrations different than those found on the ovarian surface, which may in turn increase their tendency to neoplasia (Auersperg *et al.*, 1998).

1.6.3. The gonadotropin theory

Cramer and Welch (1983) have also proposed that gonadotropins may be implicated in the pathogenesis of ovarian cancer either by directly acting on OSE or by regulating the production or release of growth factors by other cells within the ovary. The gonadotropin theory of ovarian tumorigenesis is based on both circumstantial and experimental evidence: ovarian cancer incidence increases with age, coinciding with the onset of menopause and the rise of pituitary gonadotropin hormones (Monroe and Menon, 1977). Pregnancy and oral contraceptives on the other hand not only decrease the number of ovulatory events in a woman's life but also lower the level of gonadotropins and may thereby reduce the risk of ovarian cancer (Cramer and Welch, 1983).

Experimental evidence includes the presence of bilateral ovarian tubular adenomas, composed of invasive epithelial tubules and interstitial cells in 100% of c-*kit* mutant mice with the genotype W^x/W^v (Murphy and Beamer, 1973). These mice also have elevated levels of gonadotropins, consistent with the increased incidence of ovarian epithelium carcinomas seen in menopausal women. Suppression of gonadotropins in these mice prevents the formation of the tubular adenomas (Blaakær *et al.*, 1995).

1.6.4. Genetic predisposition

Five to 10% of ovarian cancer cases are thought to be hereditary. Two tumour suppressors, BRCA1 (Miki *et al.*, 1994; Narod *et al.*, 1991) and BRCA2 (Wooster *et al.*, 1995; Wooster *et al.*, 1994) have been identified as breast/ovarian cancer susceptibility

genes through linkage studies within families of both ovarian and breast cancer. The biochemical functions of the proteins coded by BRCA1 and BRCA2 are thought to be transcriptional activation and they may be involved in DNA repair (Aunoble *et al.*, 2000). BRCA1 and BRCA2 germline mutation carriers have a life time risk of 40-66% and 10-20% for breast and ovarian cancer, respectively (Easton *et al.*, 1993; Easton *et al.*, 1995), and diagnosis usually occurs 10 years earlier than with sporadic ovarian cancer (Laplace-Marieze *et al.*, 1999; Shattuck-Eidens *et al.*, 1997).

Hereditary nonpolyposis colorectal cancer syndrome (Lynch II syndrome) is also thought to predispose to ovarian cancer, due to mutations in DNA mismatch repair genes (Hamilton *et al.*, 1999).

2. *Ovarian Surface Epithelial Cells*

2.1 Known Characteristics and Functions

Being mesodermally derived and relatively uncommitted, the OSE has both epithelial and mesenchymal characteristics. Thus OSE cells are capable of producing both epithelial and mesenchymal extracellular matrix proteins such as laminin, fibronectin and collagen I, III and IV (Kruk and Auersperg, 1994; Auersperg *et al.*, 1994). OSE cells may therefore be involved in rebuilding the extracellular matrix (ECM) after ovulation. The OSE is also characterized by keratin, mucin, desmosomes, apical microvilli and a basal lamina (Auersperg *et al.*, 1994).

In addition to the proteases secreted at the time of ovulation, OSE cells secrete bioactive cytokines including interleukin-1, interleukin-6, and colony stimulating factors (Lidor *et al.*, 1993; Ziltener *et al.*, 1993), and growth factors such as transforming growth

factor-α (TGF-α) (Jindal *et al.*, 1994), transforming growth factor-β (TGF-β) (Berchuck *et al.*, 1992b; Nilsson *et al.*, 2001), keratinocyte and hepatocyte growth factors (KGF and HGF) (Parrott *et al.*, 2000a; Parrott and Skinner, 2000), and Kit ligand (KL) (Parrott *et al.*, 2000b). Although some of these cytokines have been suggested to be involved in the repair of the ovulatory wound (Lidor *et al.*, 1993; Ziltener *et al.*, 1993), there is also the possibility that they may be involved in the transformation and/or progression of ovarian cancer.

Both normal, and some but not all transformed OSE cells, have also been shown to express many receptors to the above growth factors and hormones. For instance, components of the TGF-β receptor system have been shown to be expressed in normal and transformed OSE cells (Jindal *et al.*, 1995; Bartlett *et al.*, 1997; Nilsson *et al.*, 2001). Normal human OSE and some ovarian cancer cells have also been shown to express epidermal growth factor (EGF)/TGF-α receptor (Stromberg *et al.*, 1992; Berchuck *et al.*, 1991; Rodriguez *et al.*, 1991). Expression of this receptor in ovarian cancers has been associated with poor survival but not with histologic grade (Berchuck *et al.*, 1991). Finally, the Kit receptor has only very recently been reported to be expressed by normal bovine and human OSE (Parrott *et al.*, 2000b) as well as transformed hOSE cells (Parrott *et al.*, 2000b; Tonary *et al.*, 2000).

Progesterone, androgen and estrogen receptors have been shown to be expressed on rat OSE and normal and transformed human OSE (Hamilton *et al.*, 1984; Hamilton *et al.*, 1983; Karlan *et al.*, 1995b; Hillier *et al.*, 1998; Brandenberger *et al.*, 1998; Hamilton *et al.*, 1982).

Finally, receptors for both follicle stimulating hormone (FSH) and luteinizing hormone (LH) have been found on human and bovine OSE and ovarian cancer cells (Zheng *et al.*, 1996; Konishi *et al.*, 1999; Parrott *et al.*, 2001).

2.2 Growth Regulation of Ovarian Surface Epithelial Cells

OSE cells have not only been found to express receptors and ligands to many growth factors and hormones, but also respond to these factors. TGF-β has been shown to act as an inhibitory growth factor in normal OSE (Hurteau *et al.*, 1994; Berchuck *et al.*, 1992b; Ismail *et al.*, 1999) but it may lose its inhibitory ability and synergise with EGF and TGF-α after transformation (Godwin *et al.*, 1992). This loss of responsiveness observed in ovarian cancer cells may play a role in the development of ovarian cancer (Berchuck *et al.*, 1992b). EGF/TGF-α on the other hand are potent mitogens to normal and transformed OSE cells (Stromberg *et al.*, 1992; Godwin *et al.*, 1992; Siemens and Auersperg, 1988; Rodriguez *et al.*, 1991; Crew *et al.*, 1992).

Gonadotropins are known to stimulate growth of rabbit (Osterholzer *et al.*, 1985b) and bovine (Parrott *et al.*, 2001) OSE cells as well as normal and transformed hOSE cells (Konishi *et al.*, 1999; Syed *et al.*, 2001). Conversely, Ivarsson *et al.* (2001) have reported inhibition of hOSE proliferation by FSH and no effect by LH.

KGF, HGF (Parrott *et al.*, 2000a; Parrott and Skinner, 2000) and KL (Parrott *et al.*, 2000b) have all been reported to stimulate the growth of bovine and human OSE as well as OSE derived from cancer.

3. *Epithelial-Stromal Cell Interactions*

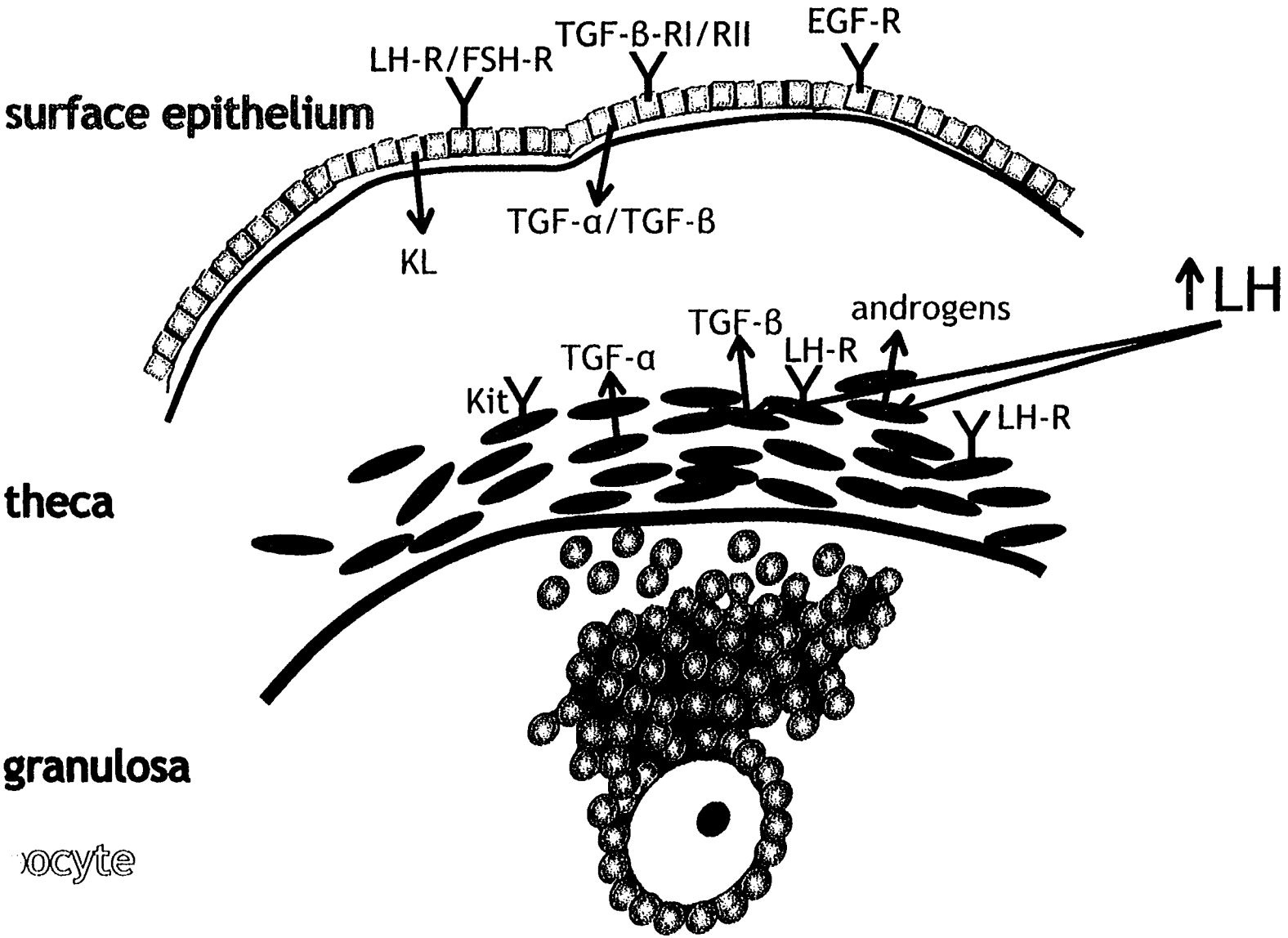
3.1 Epithelial-Stromal Cell Interactions

Epithelial-stromal interactions are one of the most common cell-cell interactions in many organs. Not only are epithelial-stromal interactions considered essential in embryologic development and in organ restructuring and wound healing in adult life (Lin and Bissell, 1993; Donjacour and Cunha, 1995), but abnormal interactions between these two cell types has been suggested to be involved in the progression of many cancers (Beresford, 1986) including breast and prostate cancer (Hayashi and Cunha, 1991; Sakakura *et al.*, 1979).

3.2 Epithelial-Stromal Cell Interactions in the Ovary

Theca-interstitial cells are known to play a role in the maintenance of structural integrity and the regulation of follicular function by providing steroid substrates for granulosa steroidogenesis and modulate granulosa cell function via paracrine factors. More recently, stromal/thecal cells have been suggested to play a role in paracrine interactions with OSE cells. The stromal/thecal cells underlying the OSE produce molecules such as growth factors that are known to modulate OSE cell growth (Figure 3). These include TGF-α (Teerds and Dorrington, 1992; Kudlow *et al.*, 1987; Lobb *et al.*, 1989), TGF-β (Skinner *et al.*, 1987; Chegini and Flanders, 1992; Mulheron *et al.*, 1991), KGF and HGF (Parrott *et al.*, 1994; Gulati and Peluso, 1997; Parrott and Skinner, 1998; Parrott *et al.*, 2000a). The production of TGF-β and the changing levels of gonadotropins at the time of ovulation are of particular interest as they may allow OSE and

Figure 3: Epithelial-stromal cell interactions in the ovary


surface epithelium
LH-R/FSH-R
TGF-β-RI/RII
EGF-R
KL
TGF-α/TGF-β
↑LH
androgens
TGF-β
LH-R
TGF-α
Kit
LH-R
theca
granulosa
ocyte

stromal/thecal cells to communicate in order to coordinate activities leading to ovulation. Mulheron *et al.* (1991) have reported the downregulation of TGF-β by hCG.

The effect of stromal-conditioned media (SCM) itself on OSE cell proliferation has also been investigated by two separate groups (Vigne *et al.*, 1994; Karlan *et al.*, 1995a), yielding conflicting results. The suggested paracrine interactions between stromal/thecal cells and OSE cells may very well play an important role in the process of ovulation and interactions gone awry may be involved in the pathogenesis of ovarian cancer.

4. Kit and KL

4.1 Structure/Function of Kit and KL

The proto-oncogene c-*kit*, which encodes the Kit transmembrane receptor at the *dominant white spotting* (*W*) locus of chromosome 5 of the mouse (Chabot *et al.*, 1988; Geissler *et al.*, 1988; Nocka *et al.*, 1989), is the normal cellular homologue of the oncogene v-*kit* of the Hardy-Zuckermann 4-feline sarcoma virus (Besmer *et al.*, 1986) and a member of the largest family of oncogenes: the tyrosine kinases. This 145 kilodalton (kD) glycoprotein is structurally related to the receptors for colony stimulating factors and platelet-derived growth factor receptors (Yarden *et al.*, 1987).

Kit ligand, also known as stem cell factor, mast cell growth factor and steel factor (Nocka *et al.*, 1990; Williams *et al.*, 1990; Zsebo *et al.*, 1990a), is encoded at the murine *steel* (*Sl*) locus (Zsebo *et al.*, 1990b) and is a multipotent growth factor important for gametogenesis, melanogenesis and haematopoiesis during development and in adult life (Russell, 1979). KL has two isoforms, which arise from alternatively spliced mRNAs

(Huang *et al.*, 1992); although both are translated into transmembrane proteins, KL-1 is proteolytically cleaved into a soluble form. The exon coding for the proteolytic cleavage site is alternatively spliced from the KL-2 transcript yielding a stable membrane-bound KL. Binding of KL to the extracellular portion of the receptor induces dimerization of the membrane receptor and activation via autophosphorylation of the cytoplasmic portion of the receptor (Figure 4). Activated Kit can phosphorylate downstream effector molecules leading to various signalling pathways resulting in cell proliferation (Tsai *et al.*, 1991; Metcalf and Nicola, 1991; Matsui *et al.*, 1991), maturation (Tsai *et al.*, 1991), migration (Godin *et al.*, 1991) and survival (Dolci *et al.*, 1991; Godin *et al.*, 1991; Pesce *et al.*, 1993; Packer *et al.*, 1995) in various cell types.

4.2 Kit and KL Expression in the Ovary

Kit and KL are expressed in the different cell types of the ovary, such that cell-cell interactions essential for follicular development in the ovary may occur (see Figure 5). Kit receptor is expressed by the oocyte (Manova *et al.*, 1990; Horie *et al.*, 1991; Motro *et al.*, 1991; Manova *et al.*, 1993; Motro and Bernstein, 1993; Horie *et al.*, 1993; Ismail *et al.*, 1997), the stromal/thecal cells (Manova *et al.*, 1990; Manova *et al.*, 1993; Motro and Bernstein, 1993; Parrott and Skinner, 1997), and OSE cells (Parrott *et al.*, 2000b) while both membrane-bound and soluble forms of KL are expressed by the granulosa cells (Motro *et al.*, 1991; Manova *et al.*, 1993; Motro and Bernstein, 1993; Laitinen *et al.*, 1995) thereby allowing paracrine interactions between the oocyte and the granulosa cells (Dolci *et al.*, 1991; Godin *et al.*, 1991; Matsui *et al.*, 1991) and the granulosa and thecal cells (Parrott and Skinner, 1997). KL has also been found in the OSE cells (Tisdall *et al.*, 1997; Ismail *et al.*, 1999; Parrott *et al.*, 2000b) suggesting that some aspect of the OSE-

Figure 4: Kit receptor

The Kit receptor is a transmembrane receptor that is activated when bound by its ligand. Binding of KL to the extracellular portion of the receptor induces dimerization of the membrane receptor and activation via autophosphorylation of the cytoplasmic portion. Activated Kit is then able to phosphorylate downstream effector molecules leading to various signaling pathways.

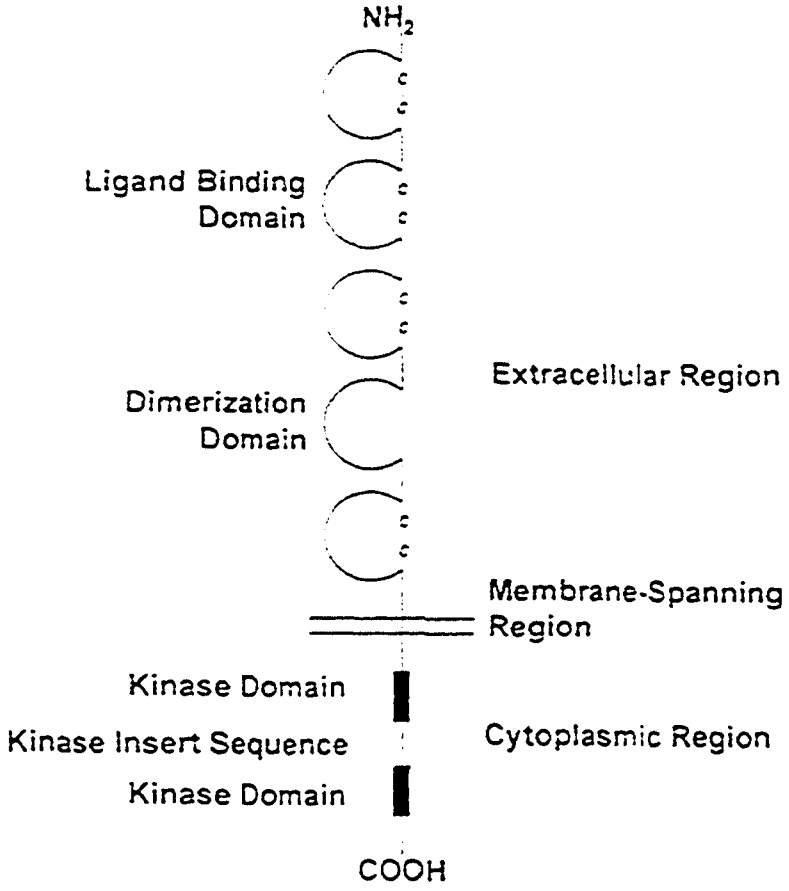
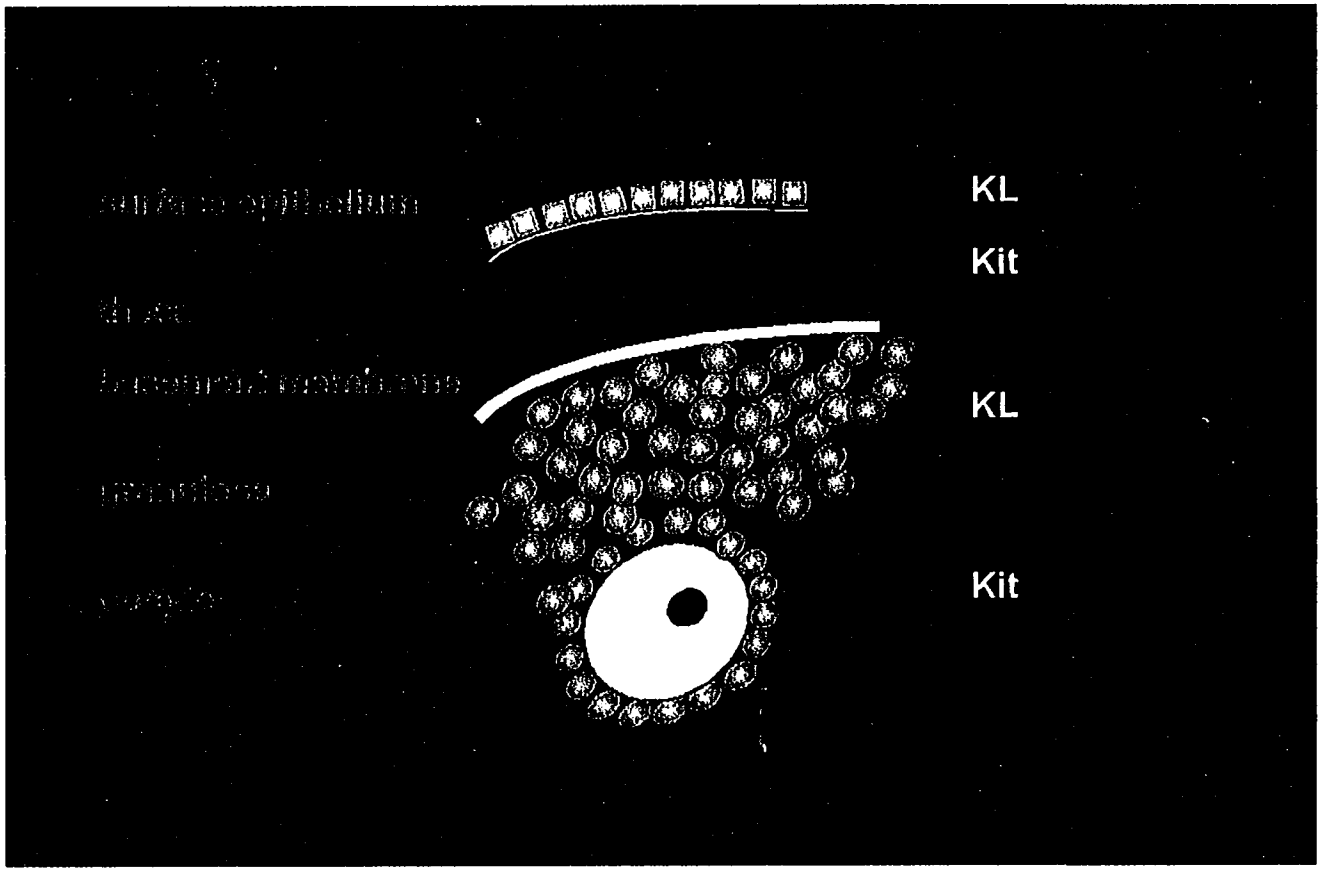

NH_2
Ligand Binding Domain
Dimerization Domain
Extracellular Region
Membrane-Spanning Region
Kinase Domain
Kinase Insert Sequence
Kinase Domain
Cytoplasmic Region
COOH

Figure 5: Expression of Kit and KL in the ovary

Kit and KL are expressed in the different cell types of the ovary in a manner permitting interaction between these different cell types.


KL
Kit
KL
Kit

thecal cell interactions may be mediated by KL activation of the Kit receptor in the underlying stromal/thecal cells

4.3 Regulation of Kit and KL Expression

Growth factors and hormones have been reported to regulate the expression of Kit and its ligand in many cell types including cells of the ovary, thereby regulating the paracrine interactions mediated by this receptor-ligand pair.

One such growth factor, TGF-β, has been shown to inhibit expression of Kit in haematopoietic progenitor cells (Dubois *et al.*, 1994; Heinrich *et al.*, 1995; Sansilvestri *et al.*, 1995), acute myelogenous leukemia blast cells (De Vos *et al.*, 1993) and colorectal carcinoma mucosa cells (Bellone *et al.*, 1997). While TGF-β has also been reported to inhibit the expression of KL in marrow stromal cells (Heinrich *et al.*, 1995) and in ROSE 199 cells (Ismail *et al.*, 1999), Nilsson *et al.* (2001) have reported the upregulation of KL in bovine OSE by this growth factor, suggesting possible species specific differences in the regulation of the ligand for the Kit receptor. Production of TGF-β in the ovary by both OSE and thecal cells may therefore not only regulate the proliferation of these cells but also the expression of Kit and KL by these cells. Accordingly, Heberlein *et al.* (1999) have reported the downregulation of c-*kit* in haematopoietic progenitors by stromal cells, supporting the suggestion of similar interactions in the ovary.

The fluctuating levels of FSH and LH during the ovarian cycle are not only known to exert direct effects on cells of the ovary but also to regulate the expression of Kit and KL in the different cell types of the ovary, thereby influencing the interactions between these cells, especially around the time of ovulation. Both FSH and LH/human chorionic gonadotropin (hCG) have been shown to decrease Kit expression in theca cells (Motro

and Bernstein, 1993) and oocytes (Horie *et al.*, 1991) of mouse ovaries, and increase KL expression in rat (Ismail *et al.*, 1996) and mouse (Motro and Bernstein, 1993) granulosa cells in vivo and bovine OSE in vitro (Parrott *et al.*, 2001). However, both gonadotropins have also been reported to decrease KL expression in cultured human granulosa cells (Laitinen *et al.*, 1995), further emphasizing the possibility of species-specific differences.

The modulation of the expression of this receptor-ligand pair by cyclic adenosine monophosphate (cAMP), the intracellular mediator of FSH and LH, has also been investigated in various cell types: cAMP increases Kit in F9 mouse teratocarcinoma cells (Nishina *et al.*, 1992), and erythroleukemia cells (Ogawa *et al.*, 1995), and increases KL in mouse granulosa cells (Packer *et al.*, 1994), in ROSE 199 cells (Ismail *et al.*, 1999) and mouse Sertoli cells (Rossi *et al.*, 1993; Tajima *et al.*, 1993). Additionally, studies done on various cell types have shown that Kit expression is decreased by tumour necrosis factor alpha (Khoury *et al.*, 1994), erythroid differentiation factor/activin A (Hino *et al.*, 1995), PKC activation (Asano *et al.*, 1993; Ogawa *et al.*, 1995), and both Kit and KL expression are downregulated by inflammatory stimuli (Koenig *et al.*, 1994; Konig *et al.*, 1997).

4.4 Kit and Cancer

The loss, or gain of Kit function or expression as well as co-expression of Kit and its ligand in a variety of cancer cell types have been implicated in the pathogenesis of many human cancers (Arber *et al.*, 1998) including: melanoma (Lassam and Bickford, 1992; Natali *et al.*, 1992a; Natali *et al.*, 1992c); lung (Sekido *et al.*, 1991; Hibi *et al.*, 1991; Sekido *et al.*, 1993; Natali *et al.*, 1992c); thyroid (Natali *et al.*, 1995); breast (Hines *et al.*, 1995; Chui *et al.*, 1996; Natali *et al.*, 1992b; Natali *et al.*, 1992c); testicular (Strohmeyer *et al.*, 1991; Natali *et al.*, 1992c); gynaecological tumours (Inoue *et al.*,

1994); ovarian (Tonary *et al.*, 2000; Natali *et al.*, 1992c); and gastrointestinal tumors (Sakurai *et al.*, 1999; Bellone *et al.*, 1997).

4.5 Kit in Ovarian Cancer

Although one group has recently reported expression of both Kit and KL in normal OSE cells (Parrott *et al.*, 2000b), most have reported that normal OSE cells express KL but not Kit (Inoue *et al.*, 1994; Ismail *et al.*, 1999; Tonary *et al.*, 2000). There has also been accumulating evidence that ovarian cancer cells and tumours express Kit (Arber *et al.*, 1998; Natali *et al.*, 1992c) or co-express Kit and its ligand (Inoue *et al.*, 1994; Tonary *et al.*, 2000), suggesting a possible role for Kit and KL signalling in ovarian tumorigenesis. Tonary *et al.* (2000) have reported frequent expression of Kit in epithelial invaginations and inclusion cysts as well as in early stage tumours, but not in normal OSE (see Figure 6). This group further reported that patients with tumours that do not express Kit have shorter disease-free survival time than those whose tumours express Kit. Taken together, these findings suggest that c-kit might play a role in early tumorigenesis, and that loss of c-kit expression may be associated with poor prognosis. This decrease in Kit expression in later stage tumours has also been reported by Parrott *et al.* (2000b) and association with loss of Kit expression and tumour progression has also been observed in other types of cancers (Natali *et al.*, 1992a).

5. ROSE 199 Cell Line and Primary ROSE and Stromal Cells

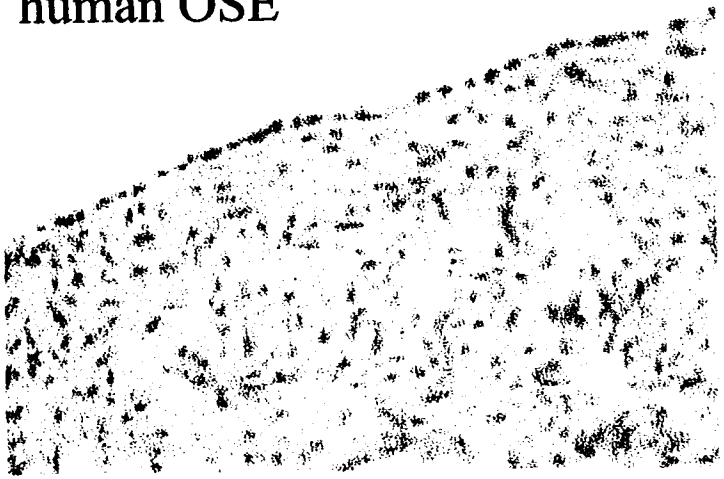
5.1 ROSE 199 Cell Line

The ROSE 199 cell line, which was used in the preliminary experiments of this study, was derived from immortalized yet non-tumorigenic ROSE

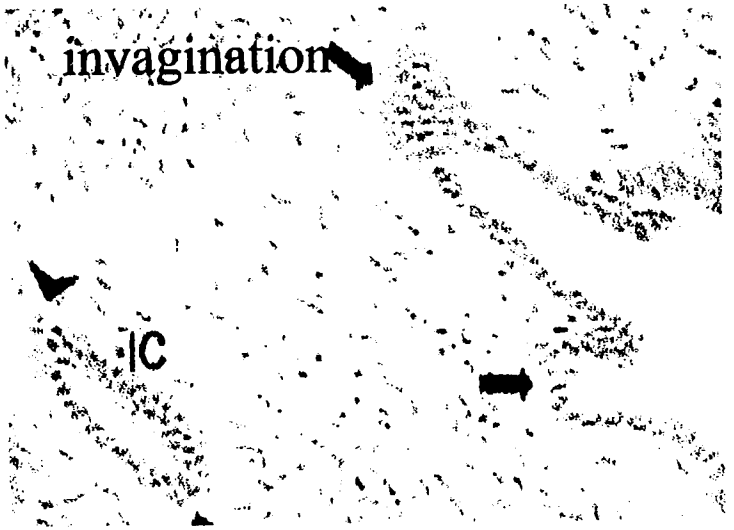
Figure 6: Expression of KIT protein in ovarian epithelial cells of inclusion cysts and ovarian tumours

Immunohistochemical staining for KIT in human ovaries has shown that while normal human OSE cells do not express KIT receptor. OSE in invaginations, inclusion cysts and in ovarian cancers can express KIT receptor.

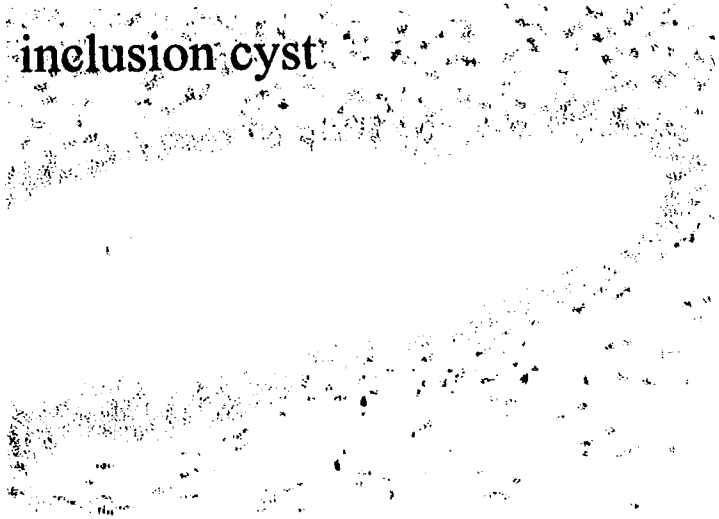
human OSE



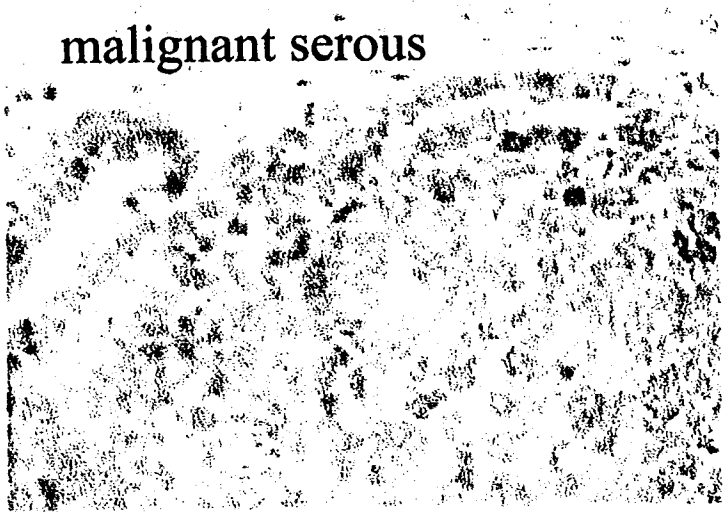
invagination



inclusion cyst



malignant serous



(Adams and Auersperg, 1985). These cells have been shown to retain their epithelial morphology in subconfluent cultures but crowded and late passage cultures of ROSE 199 cells exhibit ovarian tumour-like appearance and behaviour. ROSE 199 therefore provides a good model to study the neoplastic transformation of the ovarian surface epithelium (Adams and Auersperg, 1985).

Like hOSE (Auersperg *et al.*, 1994), ROSE 199 cells also express ECM proteins such as laminin, fibronectin and collagen types I and III which may enable the ROSE cells to participate in the post-ovulatory re-building of the ECM (Kruk and Auersperg, 1994). Stromal-like activities such as high collagen production may contribute to the neoplastic characteristics of OSE (Adams and Auersperg, 1985).

As with many other normal OSE cells (Hurteau *et al.*, 1994; Berchuck *et al.*, 1992b), TGF-β was shown to reduce the proliferation of ROSE 199 cells but this inhibition was not associated with increased cell death (Ismail *et al.*, 1999). Additionally KL but not Kit messenger ribonucleic acid (mRNA) was found to be expressed in ROSE 199 cells, mostly in its soluble form, and TGF-β reduced KL mRNA expression in these cells (Ismail *et al.*, 1999). Nilsson *et al.* (2001) on the other hand have reported an opposite effect of TGF-β on bovine OSE.

This cell line has also been used successfully in transfection experiments with erb-B2/neu, which induced transformation in vitro and in vivo (Davies *et al.*, 1998).

5.2 Primary ROSE Cell Cultures

Adams and Auersperg (1981) have described a method to isolate and culture primary ROSE cells which retain their original morphology after 10 months and 36 passages in culture (Adams and Auersperg, 1981), in contrast with hOSE cells which tend

to assume atypical morphologies in culture (Auersperg *et al.*, 1994). Cultured ROSE cells have a characteristic epithelial morphology and express the epithelial marker keratin, which distinguishes them from other ovarian cell types (Adams and Auersperg, 1981). Cultured primary ROSE cells have also been reported to have basement membranes, microvilli and apical intercellular junctions (Adams and Auersperg, 1981), express ovarian steroid receptors (Hamilton *et al.*, 1982) and KL (Ismail *et al.*, 1999). While both steroidogenic factor-1(Nash *et al.*, 1998) and TGF-β (Ismail *et al.*, 1999) are normally growth inhibitory to ROSE cells, TGF-β may lose its inhibitory ability and synergise with EGF and TGF-α after transformation (Godwin *et al.*, 1992). EGF and TGF-α on the other hand are known to be potent mitogens to normal and transformed ROSE cells (Godwin *et al.*, 1992).

ROSE cells subjected to repeated subculturing in vitro undergo spontaneous transformation acquiring features such loss of contact inhibition, the capacity for substrate-independent growth, tumorigenicity and cytogenic abnormalities (Godwin *et al.*, 1992; Testa *et al.*, 1994). This capacity for malignant transformation of ROSE cells following repeated growth in vitro has therefore been exploited and primary ROSE cells have been used as a model for epithelial ovarian cancer (Abdollahi *et al.*, 1997).

Normal OSE of rat, rabbit, human and bovine have been cultured (Skinner *et al.*, 1987; Adams and Auersperg, 1981; Osterholzer *et al.*, 1985b; Siemens and Auersperg, 1988). Although human OSE cultures may be most appropriate in the study of ovarian cancer, they are difficult to obtain in sufficient amounts and in suitably pure form for in vitro experimentation and because they consist of a single layer of cells they can be easily damaged during handling. The growth of hOSE is also known to be limited and

unpredictable, in that these cells invariably undergo degeneration or senescence after a few weeks (Siemens and Auersperg, 1988).

Fresh rat ovaries on the other hand are very accessible and the method used to isolate them allows for very pure cultures, which can be maintained for long periods (Adams and Auersperg, 1981). Primary cultures of ROSE cells and their ability to spontaneously transform therefore make a suitable epithelial ovarian cancer model due to their greater accessibility, stability and reproducibility. ROSE cells also make for a great model due to the commercial availability of an immortalized yet non-tumorigenic cell line, ROSE 199.

5.3 Primary Rat Ovarian Stromal/thecal Cell Cultures

Primary ovarian stromal/thecal cell cultures have been used by many to investigate activities and functions such as differentiation, steroidogenic capability and growth regulation (Zachow *et al.*, 1993; Duleba *et al.*, 1997; Magoffin and Erickson, 1982b; Magoffin and Erickson, 1981; Magoffin, 1989; Schwartz and Roy, 1998). Over the years, two principal methods for isolation of stromal/thecal cells have emerged: microdissection which has been used by those working with animals with large size ovaries such as bovine (Vigne *et al.*, 1994) and human (Karlan *et al.*, 1995a) and Percoll density gradient which is more efficient when working with smaller size ovaries from animals such as rat and hamster (Magoffin and Erickson, 1982a; Schwartz and Roy, 1998). This second method is based on the fact that granulosa cells being steroidogenic are less dense than undifferentiated stromal/thecal cells, which are fibroblast like in appearance. Because of the relative ease and available knowledge on the isolation of

stromal/thecal cells, rat stromal/thecal cells were isolated following the Percoll density gradient method and used to generate SCM during this project.

6. *Rationale*

6.1 Importance of Chosen Problem

Although ovarian cancer is rare, it is the most deadly of gynaecological cancers. Unfortunately, still very little is known about the cells that give rise to 90% of ovarian cancers, the OSE cells, and much of the available data remains controversial. One very popular theory regarding the etiology of ovarian cancer is Fathalla's incessant ovulation hypothesis. Repeated ovulation may not only increase the number of opportunities for malignant transformation of OSE cells but also increase the number of inclusion cysts formed during the repair of the ovulatory wound, which are thought to be sites of origin of ovarian tumours (Cramer and Welch, 1983). This entrapment of normal or transformed OSE cells within the ovarian cortex, surrounded by stromal/thecal cells, may expose them to factors to which they are not normally exposed, or to concentrations different than those found on the ovarian surface, which may in turn increase their tendency to neoplasia (Auersperg *et al.*, 1998).

As described above, normal and transformed OSE cells as well as stromal/thecal cells, express many growth factors and hormones, including EGF/TGF-α, TGF-β, and KL, as well as the receptors to growth factors and hormones. It has therefore been proposed that many of these receptor/ligand pairs may play a role in ovarian cancer progression either through aberrant differentiation or increased proliferation via paracrine and/or autocrine interactions. The production of TGF-β and the changing levels of

gonadotropins at the time of ovulation are of particular interest as they may allow OSE and stromal/thecal cells to communicate in order to coordinate activities leading to ovulation.

The co-expression of Kit and KL has been implicated in the pathogenesis of many human cancers (Hibi *et al.*, 1991; Inoue *et al.*, 1994; Hines *et al.*, 1995; Tonary *et al.*, 2000). While normal OSE cells express only KL (Inoue *et al.*, 1994; Tisdall *et al.*, 1997; Ismail *et al.*, 1999; Tonary *et al.*, 2000), OSE cells in inclusion cysts and ovarian tumours have been found to express Kit receptors as well (Inoue *et al.*, 1994; Arber *et al.*, 1998; Tonary *et al.*, 2000; Natali *et al.*, 1992c). Determining what induces Kit expression under these circumstances and what its function is in OSE may help elucidate its role in ovarian tumorigenesis.

The investigation of the mechanisms regulating normal OSE cell behaviour and OSE- stromal/thecal cell interactions is critical as improved knowledge of these mechanisms may contribute to our understanding of ovulation and the formation of inclusion cysts that give rise to ovarian epithelial carcinomas. This project was therefore designed to investigate the growth regulation of OSE cells by stromal/thecal-derived factors, specifically EGF, TGF-α and TGF-β, as well as the regulation of Kit and KL mRNA expression in these cells by these factors. The effects of gonadotropic hormones, including hCG and FSH, on both of these parameters were also investigated to simulate the environment during the ovulatory process. hCG is a homologue of the pituitary gonadotropin LH, which is synthesized by the placenta. These two hormones share the same receptor and exert identical biological effects on the cells expressing LH receptors.

The ROSE 199 cell line was used in the preliminary stages of the project. However, recognizing the limitations in using an immortalized cell line, primary cultures of ROSE cells were subsequently established and used for the remainder of the project.

To further simulate the in vivo environment, exogenous stromal/thecal-derived factors were replaced by SCM, generated from primary cultures of stromal/thecal cells.

6.2 Hypotheses

The working hypotheses for the present project were as follow:

1. Rate of proliferation of OSE cells is modulated by stromal/thecal cells
2. The LH surge which induces ovulation alters the interactions between OSE and stromal/thecal cells in a way that contributes to the ovulatory process
3. Improper interactions between OSE and stromal/thecal cells may lead to predisposition to inclusion cysts or aberrations leading to ovarian cancer

6.3 Objectives

The specific objectives of this project were:

1. To investigate growth regulation of OSE by stromal/thecal-derived factors and gonadotropins
2. To investigate regulation of Kit and KL expression in OSE by stromal/thecal-derived factors
3. To investigate the effects of LH in modifying the OSE- stromal/thecal cells interactions

MATERIALS AND METHODS

Overview

This project was designed to investigate the regulation of OSE cell proliferation and of c-*kit* and KL mRNA expression in OSE cells by stromal/thecal-derived factors. The immortalized cell line ROSE 199 was chosen for the preliminary proliferation experiments to establish an in vitro model. ROSE 199 cells were cultured and treated with stromal/thecal-derived factors such as TGF-β, TGF-α and EGF. The effects of these factors on cell growth were examined by counting cells.

Recognizing the limitations of an immortalized cell line such as ROSE 199, primary cultures of ROSE cells were established and treated with the above stromal/thecal-derived factors as well as with gonadotropins. To further mimic the in vivo environment, SCM media generated from primary cultures of stromal/thecal cells was used to treat primary ROSE cells in replacement of the exogenous growth factors. The effects of these factors as well as of the SCM on ROSE cell growth, c-*kit* and KL mRNA expression were examined in primary ROSE cells by counting cells and by Northern blot and RT-PCR, respectively.

Finally an attempt was made to identify the mediator in SCM responsible for inhibiting ROSE cell proliferation.

7. Tissue Culture

7.1 Maintenance of ROSE 199 Cells in Culture

Cultures of ROSE 199 cells, a spontaneously immortalized cell line of ROSE (Adams and Auersperg, 1985), were maintained semi-confluent at 37°C and 5% CO_2 atmosphere in 100 mm tissue culture dishes (100 x 20 mm, Nalge Nunc International, Denmark) in α-minimal essential medium with phenol red dye (α-MEM; Gibco BRL, Burlington, ON.), supplemented with 10% heat-inactivated fetal calf serum (FCS; CanSera, Rexdale, ON). Cells were passaged every 2-4 days prior to reaching confluency, by first washing the cells with phosphate buffered saline (PBS) and then incubating them in 0.025% trypsin weight/volume (w/v) and 1 mM ethylenediaminetetraacetic acid (EDTA) in PBS for 2-3 minutes. Following trypsin-EDTA inactivation, cells were centrifuged at 3000 rotations per minute (rpm) for 4 minutes. Pelleted cells were resuspended in fresh media and cell number was determined using a haemocytometer.

Stocks of cells were regularly frozen by resuspending pelleted cells in α-MEM with 10% dimethyl sulfoxide (DMSO; Sigma). Cells were aliquoted at 1.0-2.0 x 10^6 cells/ml into cryogenic vials (Nalge Nunc International) and transferred on dry ice to a -80°C freezer for short term storage. For storage longer than a few weeks, frozen aliquots were transferred to liquid nitrogen.

Vials of frozen cells were thawed in a 37°C water bath, diluted 1:10 in a 15 ml Falcon tube containing warm culture medium, and centrifuged at 3000 rpm for 4 minutes. Pelleted cells were resuspended in fresh culture medium and transferred to a 60 mm tissue culture dish (60 x 15 mm, Nalge Nunc International).

7.2 Establishment of Primary Cultures of ROSE and Stromal/Thecal Cells

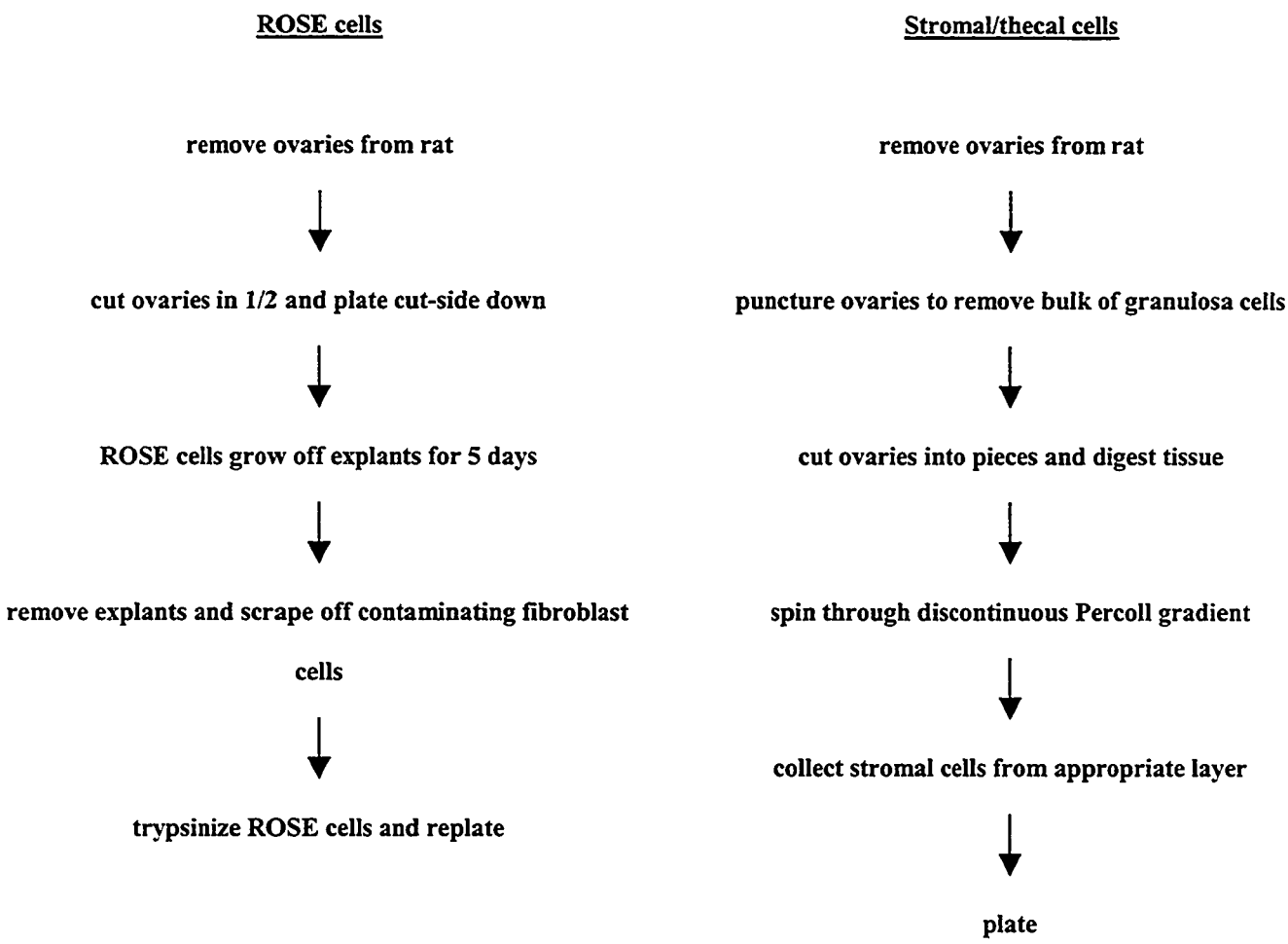
The major steps followed to isolate ROSE and stromal/thecal cells have been summarized in Figure 7

7.2.1. Isolation and maintenance of primary ROSE cells

Primary cultures of ROSE cells were established from rat ovary explants as described by Adams and Auersperg (1981), with slight modifications by Ismail *et al.* (1999). Mature lactating female Sprague-Dawley rats were obtained from Charles River Canada. Anesthetized animals were sacrificed by cervical dislocation and the ovaries were removed aseptically, carefully cleaned of extraneous tissue and sectioned on both sides laterally with respect to the hilar region. The explants were placed cut-side down on a 35 mm culture dish (35 x 10 mm Nalge Nunc International) and allowed to dry for 7 minutes before adding 2 ml of Waymouth MB 752/1 medium (Sigma) supplemented with 25% fetal bovine serum (FBS, Hyclone, Logan, UT), 100 IU/ml penicillin G (Sigma), 100 µg/ml streptomycin (Sigma), and 1.25 µg/ml fungizone (Gibco BRL). The explants were incubated at 37°C, in 5% CO_2 and 95% air for 5 days with a change of medium on Day 4. After 5 days, an outgrowth 2 to 4 mm wide of 2 to 3 x 10^4 epithelial cells surrounded the explants. First-passage cultures were prepared by removing the explants, scraping off any contaminating cells with a 25G needle, rinsing the OSE cells twice with Hank's Balanced Salt Solution (HBSS, Gibco BRL) and trypsinizing them with 0.25% trypsin in PBS for 20 minutes at room temperature. First-passage cultures were set up in 16 mm wells (Nalge Nunc International) at a density of 5000 cells/well. Primary ROSE cultures were

Figure 7: Isolation of primary ROSE and stromal/thecal cells

Major steps in the isolation of primary rat OSE and stromal/thecal cells as detailed in the text.


ROSE cells
remove ovaries from rat
cut ovaries in 1/2 and plate cut-side down
ROSE cells grow off explants for 5 days
remove explants and scrape off contaminating fibroblast cells
trypsinize ROSE cells and replate
Stromal/thecal cells
remove ovaries from rat
puncture ovaries to remove bulk of granulosa cells
cut ovaries into pieces and digest tissue
spin through discontinuous Percoll gradient
collect stromal cells from appropriate layer
plate

passaged as described above for ROSE 199 cells and maintained in 60 mm dishes (60 x 15 mm Nalge Nunc International) dishes at 37°C and 5% CO_2 atmosphere, for a maximum of 13 passages for experimental purposes.

7.2.2. Isolation and maintenance of primary stromal/thecal cells

Primary cultures of rat ovarian stromal/thecal cells were established from cells purified by discontinuous Percoll gradient as described by Magoffin (Magoffin, 1993; Magoffin, 1998), except that diethylstilbestrol (DES)-primed rats were used (Duleba *et al.*, 1997), instead of hypophysectomized rats. Immature (20-25 days old) female Sprague-Dawley rats (Charles River Canada) were injected subcutaneously each day with 0.1 ml of 10 mg/ml DES (Sigma) in sesame oil for 3 days to stimulate ovarian development. Anesthetized, animals were sacrificed on Day 4 and the ovaries dissected free of bursa and oviduct, and placed in ice-cold Medium 199 (Sigma) with HBSS supplemented with 0.35 mg/ml $NaHCO_3$, 25 mM Hepes (Sigma), and 1 mg/ml bovine serum albumin (BSA, Sigma) (H-199B). Most granulosa cells were removed by puncturing the follicles with a sterile fine-gauge hypodermic needle. The remaining ovarian tissues were used to obtain the stromal/thecal preparations. Briefly, ovarian tissues were cut into 4 to 6 pieces, washed in Medium 199 and then enzymatically digested in 0.1 ml/ovary of collagenase-DNAse solution [4 mg/ml collagenase Type I (Worthington Biochemical Co., Freehold, NJ), 10 μg/ml DNAse I (Sigma) and 10 mg/ml BSA in Medium 199]. Incubations were carried out with agitation in a 37°C water bath for a total of 90 minutes, flushing tissues through Pasteur pipets with successively smaller orifices every 30 minutes. The dispersed cells were then centrifuged at 250g for 5 minutes, resuspended in McCoy's 5a medium (Gibco BRL) and purified using a

discontinuous Percoll gradient. As depicted in Figure 8, the discontinuous Percoll density gradient was prepared by layering sequentially 1 ml of 1.06 g/ml Percoll diluted in H-199B, 2 ml of 1.055 g/ml Percoll and 2 ml of cell suspension. The gradient was centrifuged at 450g for 20 minutes at 4°C. The stromal/thecal cells were collected by aspiration from the d=1.055 layer. Purified stromal cell were cultured for a maximum of 14 days in serum-free McCoy's 5a medium supplemented with 2 mM L-glutamine (Sigma), 1 mg/ml BSA, 10 000 IU/ml penicillin, and 10 000 IU/ml streptomycin, at 37°C and 5% CO_2, 95% air, with a change of medium every 2-3 days. Viability was determined using the trypan blue exclusion test.

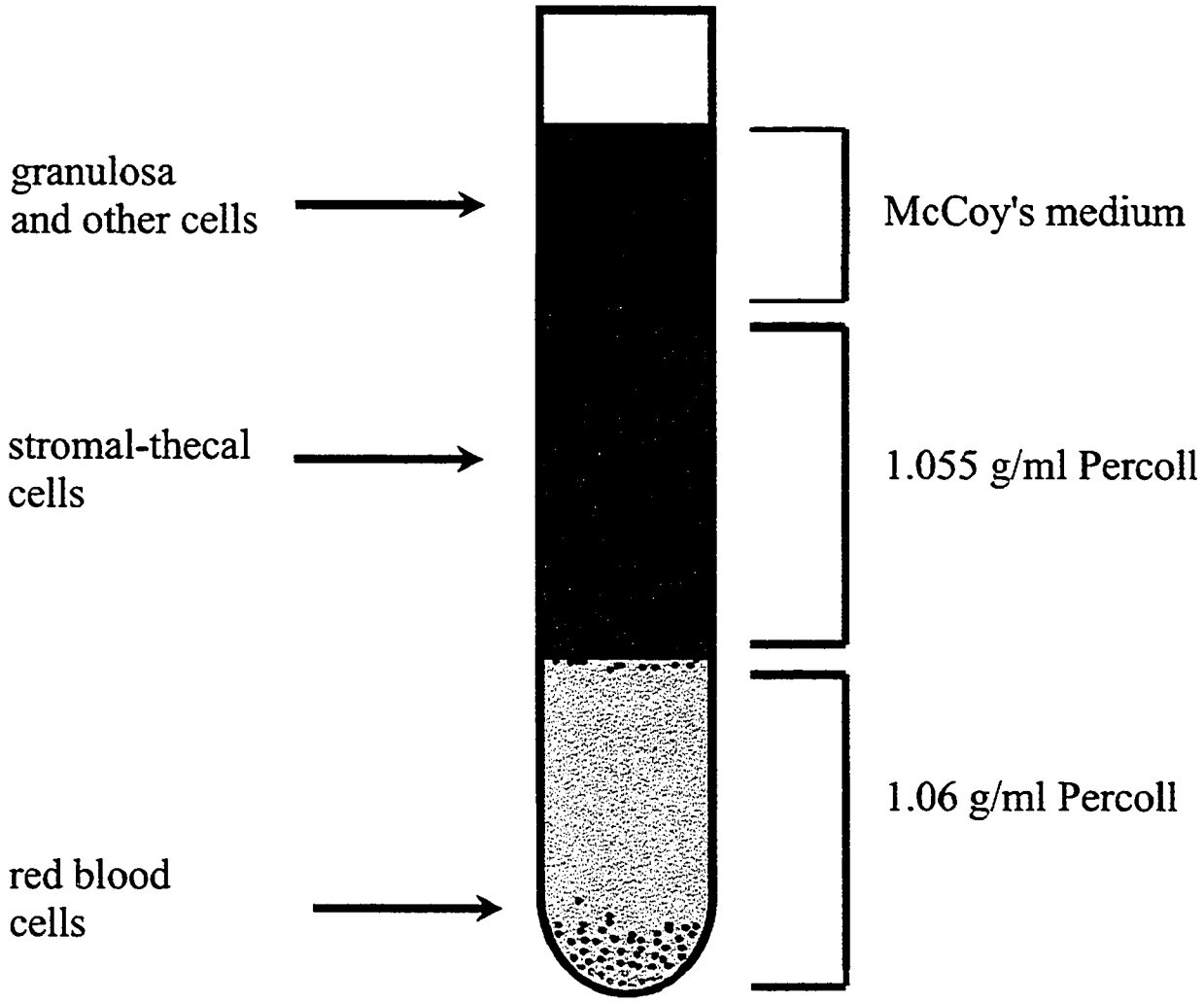
7.2.3. Immunolocalization of keratin and vimentin in ROSE and stromal/thecal cells

The purity of ROSE and stromal cell preparations was confirmed by characteristic epithelial morphologic features and keratin expression in the case of ROSE cells and vimentin expression in stromal/thecal cells. For the immunocytochemistry assay procedure, 5 X 10^4 ROSE cells and 2 X 10^4 stromal/thecal cells were cultured on 0.15% gelatin coated coverslips in 35 mm dishes. After a 24-hour incubation period at 37°C, the cells were rinsed twice in 1X Stockholm's PBS (S-PBS) and fixed in ice-cold methanol at -20°C for 5 minutes. The coverslips were allowed to air dry for 5 minutes before being rehydrated in S-PBS for 15 minutes. All antibody incubations were carried out in a humidified chamber at room temperature.

Epithelial cells were identified using the mouse monoclonal anti-cytokeratin AE1/AE3 antibody (Boehringer Mannheim, Germany), diluted 1:10 in S-PBS, 1% BSA, 0.1% sodium azide and 0.2% Triton X-100. AE1 recognizes the 56.5, 50, 50', 48 and

Figure 8: Discontinuous Percoll gradient purification of stromal/thecal cells

Stromal/thecal cells migrate into the 1.055 g/ml Percoll layer while the granulosa cells and the majority of other cell types accumulate at the top of the 1.055 g/ml Percoll layer. Red blood cells pellet at the bottom of the tube. Adaptation from (Magoffin, 1993; Magoffin, 1998)


granulosa
and other cells
stromal-thecal
cells
red blood
cells
McCoy's medium
1.055 g/ml Percoll
1.06 g/ml Percoll

40 kD cytokeratins of the acidic subfamily while AE3 recognizes all members of the basic subfamily. Following a 1 hour incubation with the anti-cytokeratin antibody, the cells were incubated for another hour with biotinylated anti-mouse immunoglobulin G (IgG) (Amersham Life Science, Arlington Heights, IL) and then for 30 minutes in Fluorescein Streptavidin (Amersham Life Science), both diluted 1:200 in S-PBS. All incubations were done at room temperature in a humidified chamber and the last incubation was protected from light. Each of these incubations was followed by 3 x 5 minute washes in S-PBS. A negative control was done by omitting the primary antibody from the procedure. The cells were then counterstained for 15 minutes with Hoechst diluted 1:10 000 in S-PBS, followed by 2 x 5 minute S-PBS washes. Finally, the coverslips were mounted with anti-fade medium (10% p-phenylendiamine, 50% glycerol and 40% PBS) onto microscope slides.

Stromal cells were identified using the mouse monoclonal anti-vimentin antibody (clone V9) (Boehringer Mannheim), diluted 1:4 in the above antibody diluent. Following a 1 hour incubation with the anti-vimentin antibody, the cells were treated for an additional hour with a Fluorescein (FITC)-conjugated AffiniPure donkey anti-mouse IgG (Jackson Immuno Research Laboratories, Inc., West Grove, PA) diluted 1:200 in the antibody diluent. Both incubations were followed by 3 x 5 minute S-PBS washes. A negative control was done by omitting the primary antibody from the procedure. The cells were counterstained with Hoechst as described above and the coverslips mounted as described for the keratin staining.

Stained cells were observed with a Zeiss Axiophot inverted microscope (Carl Zeiss Canada Ltd, Don Mills ON), equipped with a 40X Plan Neofluar lens, a Carl Zeiss

burner and epifluorescent illumination. Images were digitally recorded with a Carl Zeiss Universal CCD Video camera using Matrox Intellicam Interactive v2.0 (Matrox, Dorval PQ). TIFF images were processed using Microsoft PhotoDraw 2000 v2.

8. *Proliferation Experiments*

8.1 General Experimental Design

In vitro cell proliferation experiments were performed with ROSE 199 and in primary cultures of ROSE cells. In general, the cells maintained as described above were trypsinized, spun down and resuspended in fresh medium in order to determine a cell count and establish the volume needed for the experiment. The appropriate number of cells was seeded into culture dishes and the cells allowed to adhere to the plate for 2 hours prior to treatment with growth factors or gonadotropins. Control groups were handled in the same manner as treatment groups but received an equal volume of medium only. Most experiments were carried out in triplicate and repeated at least three times. Regulation of cell proliferation was assessed by collecting cells and counting by haemocytometry. This was done by applying 10 µl of the cell suspension to each of the 2 counting grids of the haemocytometer. The average of the cell counts within the two central $1mm^2$ grids was used in calculating the mean for a treatment group. When fewer than 20 cells were counted in the central grids, the number of cells in the four corner squares was added to the central one and the final cell count was the average of these five squares. ROSE 199 experiments were carried out in α-MEM, while those done on primary ROSE cells were carried out in Waymouth, both media containing 10% FCS. Following cell counts, samples were pooled to extract RNA as described in section 9.1.

8.2 Specific Experimental Protocols

8.2.1. Effects of growth factors

In the first series of experiments, the regulation of ROSE 199 cell proliferation by growth factors was assessed. Cells were seeded at a density of $1.5x10^5$cells/100 mm dish and, after 2 hours of plating. were treated with 0.1, 1, 10, or 100 ng/ml of TGF-β1 (R&D Systems, Minneapolis, MN), and 1, 10, or 100 ng/ml of EGF (Boehringer Mannheim) or TGF-α (R&D Systems). The TGF-α experiment was carried out in 1% FCS instead of 10% to minimize growth stimulation by FCS and its components. Each experiment was performed three times, in duplicate, except for the TGF-α experiment, which was repeated only once. Proliferation was assessed after 48 hours by determining cell number.

A second series of proliferation experiments was performed on primary cultures of ROSE cells. Cells were seeded at a density of $7.5x10^4$ cells/60 mm dish and after 2 hours of plating were treated with 10 ng/ml of TGF-β, EGF or TGF-α. Each experiment was performed at least three times, in triplicate. Regulation of cell proliferation was assessed after 4 days by determining cell number.

8.2.2. Effects of gonadotropins

The regulation of primary ROSE cell proliferation by gonadotropic hormones (hCG or FSH) was also assessed. Primary ROSE cells were seeded at a density of $7.5x10^4$ cells/60 mm dish and after 2 hours of plating were treated with 0.5, 5, or 50 IU/ml hCG (Chorulon, Intervet International, Whitby, ON), or with 10, 100 or 150 ng/ml FSH (Folligon, Intervet International). Each experiment was performed at least three times, in triplicate. Proliferation was assessed after 4 days by determining cell number.

8.2.3. Effects of SCM

To create conditions that more closely mimic the in vivo situation, primary ROSE cells were treated with SCM, which was generated by one of two methods, as described in section 10. When SCM generated by the first method was used, primary ROSE cells were seeded at a density of $5x10^4$ cells per 16 mm well, and when the second method was used to generate SCM, cells were seeded at a density of $2.5x10^4$ cells/well. After 2 hours of plating, the cells were treated with either "unconditioned" or stromal-conditioned media. Cell proliferation was assessed by counting cells after 2, 4 or 6 days, with a change of medium every other day. To investigate the possible effects of LH in modifying the OSE-stromal cell interactions, ROSE cells were also treated with SCM that was conditioned in the presence or absence of 200 ng/ml hCG.

8.2.4. Effects of blocking TGF-β in SCM

In an attempt to identify a potential mediator of the effect seen in the SCM proliferation experiments, ROSE cells cultured in SCM were treated with anti-TGF-β neutralizing antibody. Experimental conditions were exactly as described in section 8.2.3 except that a treatment group was added in which ROSE cells were cultured in the presence of 20 ng/ml of anti-TGF-β antibody (R&D Systems).

The ability of the anti-TGF-β antibody to neutralize the activity of TGF-β was tested by carrying out a proliferation experiment where primary ROSE cells were cultured either in the presence of 10 ng/ml of TGF-β or 400 ng/ml anti-TGF-β antibody alone, or both TGF-β and anti-TGF-β antibody. Primary ROSE cells were seeded at a density of $7.5x10^4$ cells/60 mm dish and cultured for 4 days after which time proliferation was assessed by counting the number of cells with a haemocytometer.

9. *RNA Analysis*

9.1 Extraction and Quantification of RNA

The Qiagen RNeasy Mini-Kit (Qiagen Inc., Mississauga, ON) was used to extract total RNA from cells in all cell proliferation experiments. As described by the manufacturer, pelleted cells were lysed in the appropriate volume of Qiagen Lysis Buffer RLT supplemented with β-mercaptoethanol and the resulting cell lysate was either frozen until ready for extraction or processed immediately for RNA extraction. Frozen samples were thawed at 37°C for 10 minutes prior to extraction to ensure that all salts were dissolved. Cell lysates were homogenized using 20 gauge needles and then put through a series of wash and spin cycles as suggested by the manufacturer. In the final step, RNA bound to the column was eluted in 50 µl RNase-free water and the concentration of the RNA samples was determined by measuring the absorbance at λ=260/280 using a Beckman DU® 640 Spectrophotometer (Beckman Instruments Inc., Mississaga, ON). RNA samples were used in Northern blot and reverse transcription-polymerase chain reaction (RT-PCR) analyses.

9.2 Northern Analysis

9.2.1. Gel electrophoresis of RNA samples

Northern blot analyses were performed to investigate the regulation of c-*kit* and KL mRNA expression in primary ROSE cells by growth factors, gonadotropins and SCM components. Samples were prepared by aliquoting 15 µg of RNA in an eppendorf tube, precipitating it with 1ml of cold ethanol-2% potassium acetate (KOAC) and centrifuging for 20 minutes at 4°C and 14000 rpm. The ethanol was then aspirated and the pellet

resuspended in 30 µl of sample buffer (50% w/v deionized formamide, 22.5% formaldehyde, 17.5% diethylpyrocarbonate (DEPC) water, 10% 10X RNA gel buffer (200 mM MOPS, 50 mM sodium acetate·$3H_2O$, 10 mM EDTA)). Samples were heated at 65°C for 10 minutes to evaporate residual ethanol and cooled on ice for 2 minutes. Three µl of sample loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol) + 10% ethidium bromide) was added to the 30 µl sample and all 33 µl were loaded onto a denaturing 0.9% agarose gel (agarose LE, Boehringer Mannheim) dissolved in 10% gel buffer (10% 10X RNA gel buffer, 85% distilled water, 5% formaldehyde). The gel solution was poured into a 250 ml gel box and left to harden. Once the gel had solidified, 2 L of gel buffer was added to the gel box so that the gel was covered by 0.5-1 cm, and the samples loaded into the gel. The gel was run overnight at 50-60 volts (V).

9.2.2. Northern transfer and hybridization

Following the overnight run, the gel was removed from the apparatus and the RNA was irradiated with ultraviolet light (UV) to visualise position of lanes and of the 28s and 18s bands. The RNA was then transferred onto a Hybond N nylon membrane (Amersham), by capillary action overnight in 2X saline sodium citrate (SSC). Again, the RNA was UV-irradiated to visualise and mark the position of lanes and of the 28s and 18s bands on the membrane and the RNA was crosslinked by UV irradiation at 100J. The membrane was placed in a glass tube, prehybridized for 1 hour and hybridized with probes (see 9.2.3) overnight, at 42°C in 20 ml of hybridization solution (50% w/v formamide, 5X Denhardt's, 5X SSPE, 1% SDS, 250 µg/ml denatured herring sperm carrier DNA). Following hybridization, the membrane was rinsed several times in a 2X SSC/0.1% sodium dodecyl sulfate (SDS) solution and then washed 2x15 minute at room

temperature. This first series of washes was followed by 2x15 minute higher stringency washes in 0.5X SSC/0.1% SDS at 65°C. The blot was bagged and inserted into a cassette for an overnight phosphoexposure and then scanned using PhosphorImager SI (Molecular Dynamics v.4.0, Sunnyvale, CA). KL and α-tubulin band intensity was estimated using ImageQuaNT software (Molecular Dynamics v.4.2a) and the ratios of KL/α-tubulin values were standardised to control levels, arbitrarily set to 1.0

9.2.3. Probes

Blots were sequentially probed with ^{32}P-labelled cDNA probes for the human c-*KIT* (5100bp), human KL (920bp), and α-tubulin. The c-*KIT* and KL cDNAs were isolated from plasmids generously provided by Dr. Frederick Jacobson (Amgen Inc, Thousands Oaks, CA), and the α-tubulin cDNA was provided by Dr. M.W. McBurney (Ottawa, ON). 3.75-7.5 ng of cDNA were denatured by boiling to 100°C for 10 minutes, then labelled with 50 μCi of α-^{32}P-deoxy-CTP using a random primed DNA labelling kit (Boehringer Mannheim), and purified of free nucleotides by a series of centrifugation through Sephadex G-50 fine spin columns. Labelled probes were denatured by boiling to 100°C for 5-10 minutes, cooled on ice for 2 minutes and added to the hybridization solution.

9.3 RT-PCR

RT-PCR was performed to investigate whether growth factors or gonadotropins could alter the proportion of transcripts encoding soluble versus membrane-bound KL in ROSE cells. KL primers spanning the alternatively spliced exon 6 of KL mRNA were used to amplify the membrane-bound KL-2 (203 bp) and soluble KL-1 (286 bp)

transcripts. RT reactions (20 µl total volume) were performed using 1.5 µg RNA with the following components added: 1X PCR buffer (Gibco BRL; all components from Gibco unless otherwise indicated), each dNTP at 0.1 mM (Boehringer Mannheim), 0.005 µg Oligo(dT)$_{12-18}$ Primer, 7 U RNase inhibitor, 4 mM $MgCl_2$, and 200 U Superscript RT. The RT reactions were incubated at 37°C for 1 hour and then heat inactivated at 95°C for 5 minutes. PCR reactions (50 µl total volume) were performed using 5 µl of cDNA with the addition of the following components: 25 pmol each KL primer [5'primer (5'-GTA TTT TCA ATA GAT CCA TTG A-3'), 3'primer (CCA GTA TAA GGC TCC AAA AGC AA-3')], 1X PCR buffer, 1 mM $MgCl_2$, and 1 U Taq polymerase. The nucleotide positions of the 5' and 3' KL primers were bp 530-551 and bp 710-732, respectively. The following program was run on a Perkin-Elmer Gene Amp® PCR System 9600 thermal cycler to amplify the RT reaction products: 3 cycles (94, 60, and 72°C, for 5, 2, and 3 minutes respectively), 30 cycles (94, 60, and 72°C, for 1, 2, and 3 minutes respectively), and a final extension time at 72°C for 10 minutes. RNA was replaced with water in one RT-PCR reaction to serve as negative control. DNA from this particular PCR reaction was precipitated before resolving on a gel by adding 1/10 of volume sodium acetate, 2-3 volumes of ice cold 100% ethanol, and incubating on dry ice for 20 minutes. The samples were then spun at 14000 rpm and 4°C for 20 minutes. After removing the supernatant, the samples were washed with 70% ethanol and spun again for 10 minutes. Finally the DNA was resuspended in 5 µl of water and 2 µl of loading dye and then run on a 2% agarose gel at 100 V for 1 hour. The gel was then stained with ethidium bromide for approximately 20 minutes and visualized under UV light.

A c-*kit* RT-PCR reaction was also performed to further confirm the results obtained by Northern blot analysis. RT reactions (20 µl total volume) were performed using 1.5 µg RNA with the following components added: 0.005 µg Oligo$(dT)_{12\text{-}18}$ Primer, 2 U RNase inhibitor, and H_2O to 12 µl. These first components were incubated at 70°C for 5 minutes prior to adding 1X 1st buffer, 0.01 M DDT, each dNTP at 0.05 mM, incubating at 42°C for 2 minutes and finally adding 200 U Superscript RT and incubating at 42°C for an additional 60 minutes. The RT reactions were then heat inactivated at 95°C for 5 minutes. PCR reactions (50 µl total volume) were performed using 1 µl of cDNA with the addition of the following components: 20 pmol each c-*kit* primer [5'primer (5'-CCC AGA GCC CAC AAT AGA TTG-3'), and 3'primer (5'CGT TCT GTC AAA TGG GCA CTC-3')], 1X PCR buffer, 1 mM $MgCl_2$, each dNTP at 0.01 mM, and 1 U Taq polymerase. The nucleotide positions of the 5' and 3' c-*kit* primers were bp 1326-1346 and bp 1874-1898, respectively. Taq polymerase was added to the reaction after a hot start at 94°C for 5 minutes, followed by 30 cycles (94 and 45°C, at 1 and 2 minutes respectively), and a final 10 minute 72°C extension time. This amplification program was run on an Eppendorf Mastercycler gradient machine. RNA was replaced with water in one RT-PCR reaction to serve as negative control. 10 µl of sample and 2 µl of loading dye were run on a 1% agarose gel at 100 V for 1 hour. The gel was then stained with ethidium bromide for approximately 20 minutes and visualized under UV light.

10. Generation of Stromal-Conditioned Media

Primary cultures of rat stromal/thecal cells (isolated as described in section 7.2.2.) were used to generate SCM. The SCM was generated by the two methods outlined in Figure 9. The first consisted of plating 5 x 10^4 stromal/thecal cells per 16 mm well, in

Figure 9: Generation of stromal-conditioned media

Major steps followed in the two methods used to generate SCM.

Method 1:

80% confluent stromal cells + 10% FCS (or unconditioned media) → 48h SCM → 50-70 % confluent ROSE → 2 or 4 days → count cells

Method 2:

80% confluent stromal cells in serum-free media (or unconditioned media) → 72h SCM → •Spin down debris •concentrate to 10X •dilute to 1X in fresh media + 10% FCS → 50-70 % confluent ROSE → 2, 4, 6 days → count cells

McCoy's 5a medium supplemented with 2 mM L-glutamine, 1 mg/ml BSA, 10 000 IU/ml penicillin, and 10 000 IU/ml streptomycin and 10% FCS. After 48 hours the media was collected and used directly to treat ROSE cells.

To rule out the possibility of effects due to media depletion inherent to this first method of SCM generation, a second method was devised. In this second method, 2.2 $x10^6$ stromal/thecal cells were plated in 100mm dishes in serum-free McCoy's 5a medium supplemented with 2 mM L-glutamine, 1 mg/ml BSA, 10 000 IU/ml penicillin, and 10 000 IU/ml streptomycin. After 72 hours the media was collected and spun down at 3000 rpm for 4 minutes to remove cellular debris. The media was then concentrated to 10X using Centriprep YM-3 filters (Centriprep Centrifugal Filter Devices, Millipore Corporation, Bedford, MA) and then rediluted to 1X with fresh McCoy's 5a medium containing 10% FCS. To investigate the possible effects of LH in modifying the OSE stromal/thecal cell interactions, SCM was also generated by stromal/thecal cells cultured in the presence of 200ng/ml hCG.

In both methods of generating SCM, control or "unconditioned" media was generated by plating the media in dishes without cells for the same amount of time, under the same conditions and submitting them to the same procedures.

11. Statistical Analyses

Statistical analyses were conducted using GraphPad Prism software 2.0 (GraphPad Software, San Diego, CA), and using SPSS 11.01 (SPSS, Inc, Chicago, Illinois) for three-way ANOVA. The two-tailed student's t-test was used when only two groups of cell counts were being compared. Data were analysed by one-, two-, or three-way analysis of variance (ANOVA) when more than two groups were being compared.

When whole group differences were detected by ANOVA ($p<0.05$), Dunnett's Multiple Comparison Post Test was used to compare individual treatment groups with control, or Newman-Keuls Multiple Comparison Test was used to compare all pairs of groups. In all tests, significance was inferred when $p<0.05$.

RESULTS

12. *Primary Cultures of ROSE and Stromal/thecal Cells*

Primary cultures of OSE cells were isolated from rat explants. After 5 days in culture, the explants were removed and the epithelial cells maintained in culture for 13 passages. At these low passages, 100% of all primary ROSE cell cultures exhibited a typical epithelioid morphology, and were arranged in an orderly cobblestone pattern (Figure 10A), making them readily distinguishable from cultured ovarian granulosa, thecal, stromal, and luteal cells. Primary cultures of rat stromal/thecal cells on the other hand were isolated using a discontinuous Percoll gradient. Stromal/thecal cells exhibited a fibroblastic morphology throughout the cultured period (Figure 10B). The steroid hormones produced by these cells are stored in the lipid drops seen in figure 10B. As is evident in Figure 10, the morphological distinction between these two cell types was very apparent.

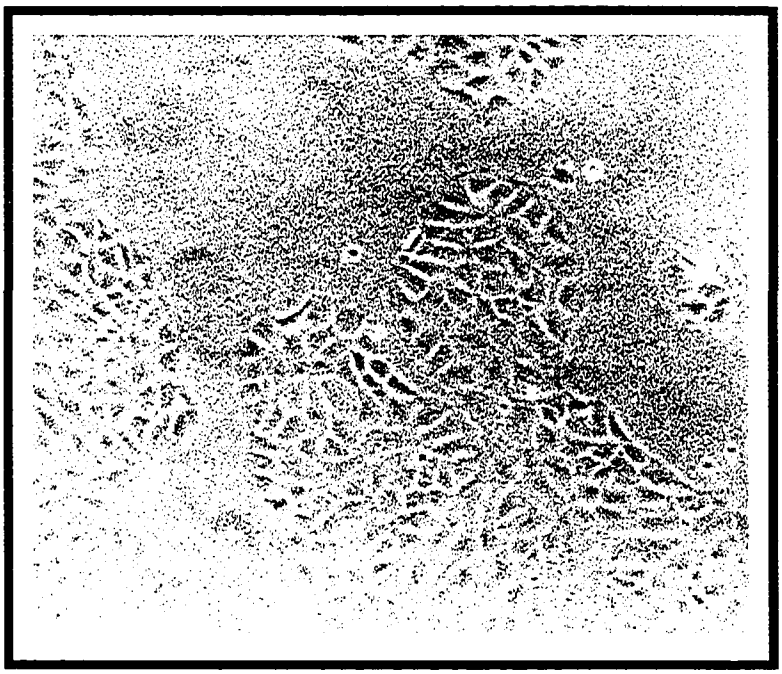
The purity of ROSE and stromal/thecal cell preparations was evaluated histochemically (Figures 11 and 12, respectively). Between 67 and 97% (mean= 78±3%, n=11) of all ROSE cell preparations examined, stained positive for keratin (Figure 11B) while none of the stromal cells stained positive (n=3), (Figure 11C). Of freshly isolated cells, 53% to 81% (68±8%, n=3) stained positive for vimentin (Figure 12B) and 100% of ROSE cells stained for vimentin also were positive (Figure 12C). No staining was observed when either primary antibody (AE1/AE3 or Clone V9) was omitted from the procedure to control for non-specific staining (Figure 11A and 12A).

Figure 10: Primary cultures of ROSE and stromal/thecal cells

Primary cultures of OSE cells (A) were isolated from rat explants. Rat ovaries were cut in half and plated cut-side down in Waymouth MB medium supplemented with 25% FCS. The explants were incubated for 5 days in 5% CO_2: 5% O_2: 90% N_2. After this period, a ring of epithelial cells was visible around each explant. The explants were removed and the epithelial cells maintained in culture for 13 passages.

Primary cultures of rat stromal/thecal cells (B) were isolated using a discontinuous Percoll gradient. Rat ovaries were punctured to remove the bulk of granulosa cells, cut into pieces and the resulting tissue was digested. The cell suspension was spun through a discontinuous Percoll gradient, the stromal/thecal cells collected from the appropriate layer and then cultured in McCoy's 5a medium for a maximum of 14 days.

A



B

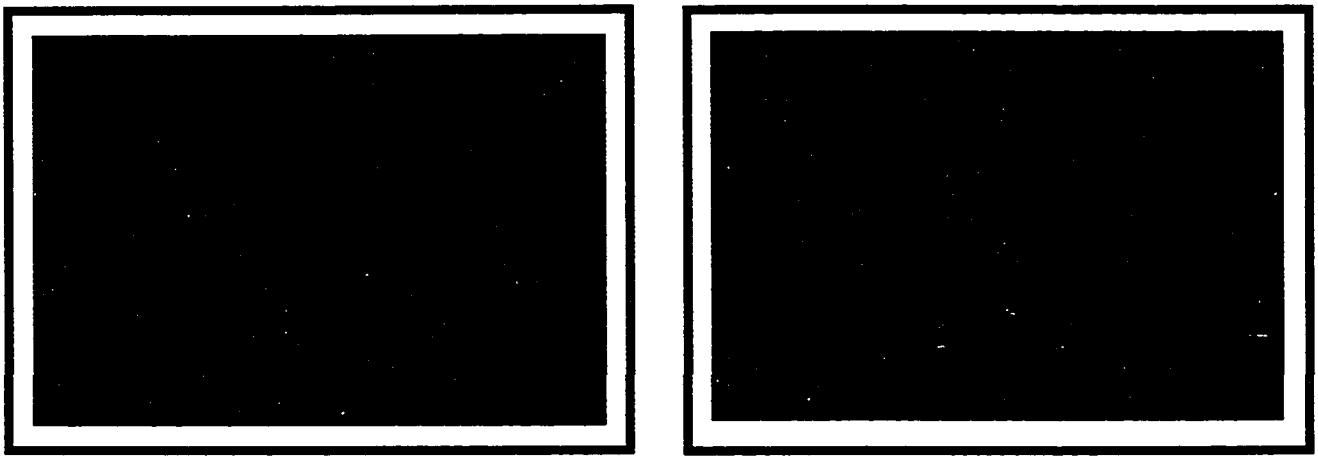
Figure 11: Immunocytochemistry for cytokeratin in primary ROSE cell

The purity of primary ROSE cell cultures was assessed by immunocytochemistry for cytokeratin. Cells were cultured on gelatin-coated coverslips in 35 mm dishes for 24 hours before being fixed in ice-cold methanol. AE1/AE3, a monoclonal antibody against cytokeratin was used to detect cytokeratin expression in the primary cultures of ROSE (B) and stromal/thecal cells (C). Cells were counterstained for DNA using Hoechst. AE1/AE3 was omitted from the procedure in A to control for non-specific staining. (40X magnification)

cytokeratin Hoechst

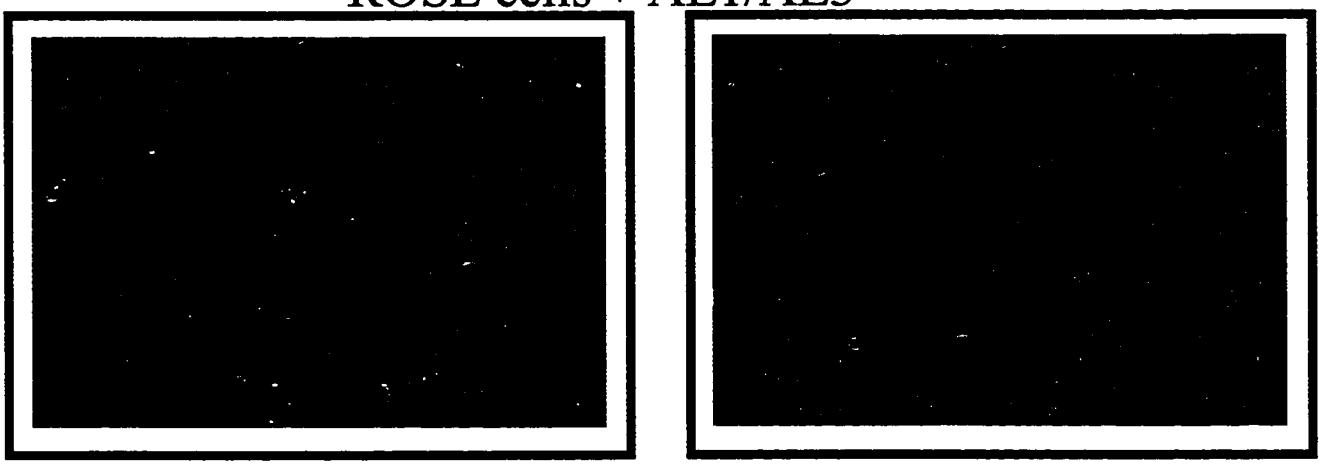
ROSE cells + no AE1/AE3

A



ROSE cells + AE1/AE3

B



stromal/thecal cells + AE1/AE3

C

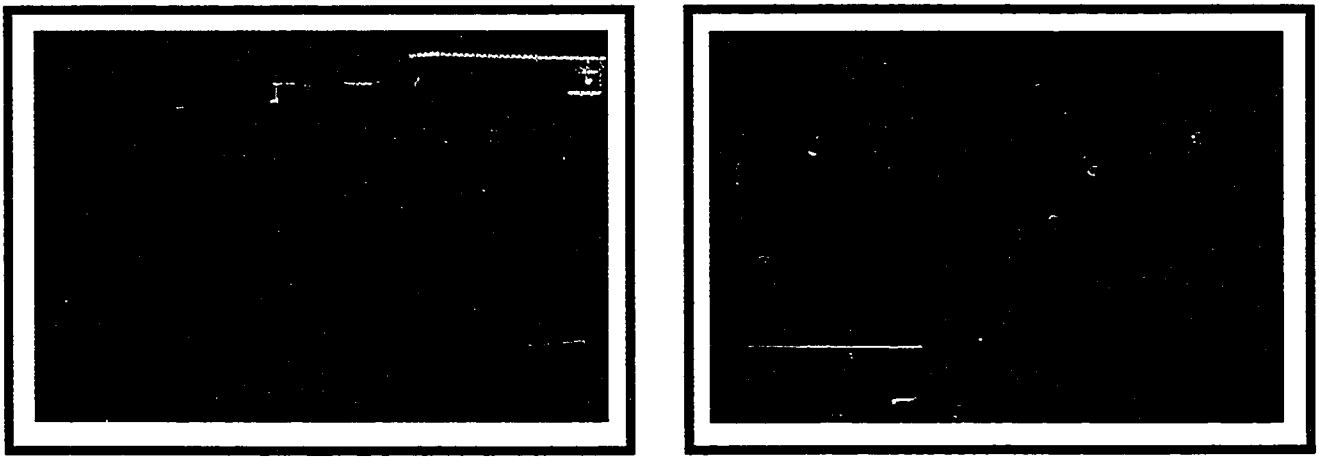


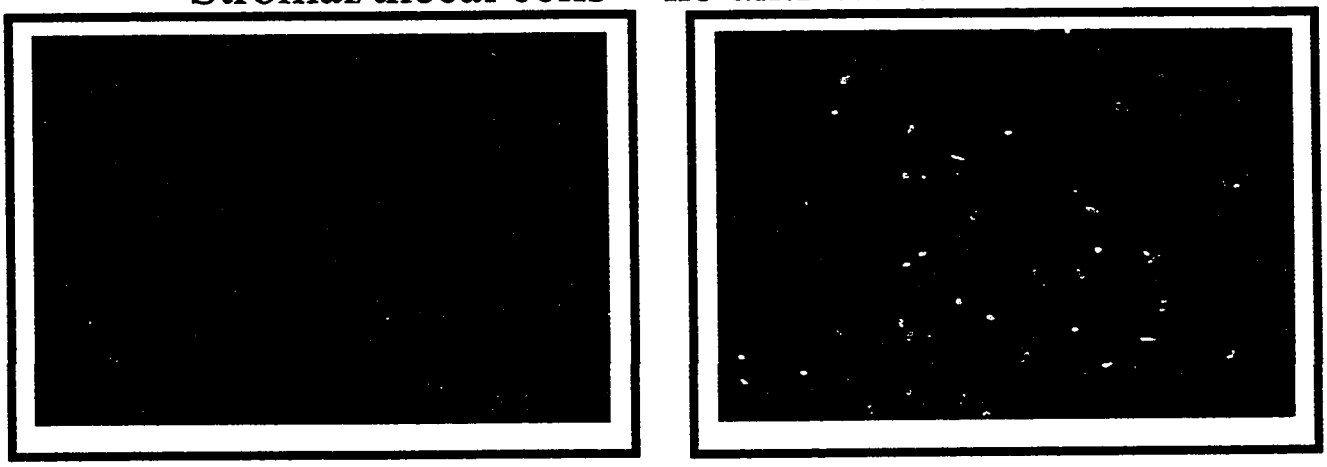
Figure 12: Immunocytochemistry for vimentin in primary stromal/thecal cells

The purity of primary thecal/stromal cell cultures was assessed by immunocytochemistry for vimentin. Cells were cultured on gelatin-coated coverslips in 35 mm dishes for 24 hours before being fixed in ice-cold methanol. Clone V9, a monoclonal antibody against vimentin was used to detect vimentin expression in the primary cultures of stromal (B) and ROSE cells (C). Cells were counterstained for DNA using Hoechst. Clone V9 was omitted from the procedure in A to control for non-specific staining. (40X magnification)

vimentin Hoechst

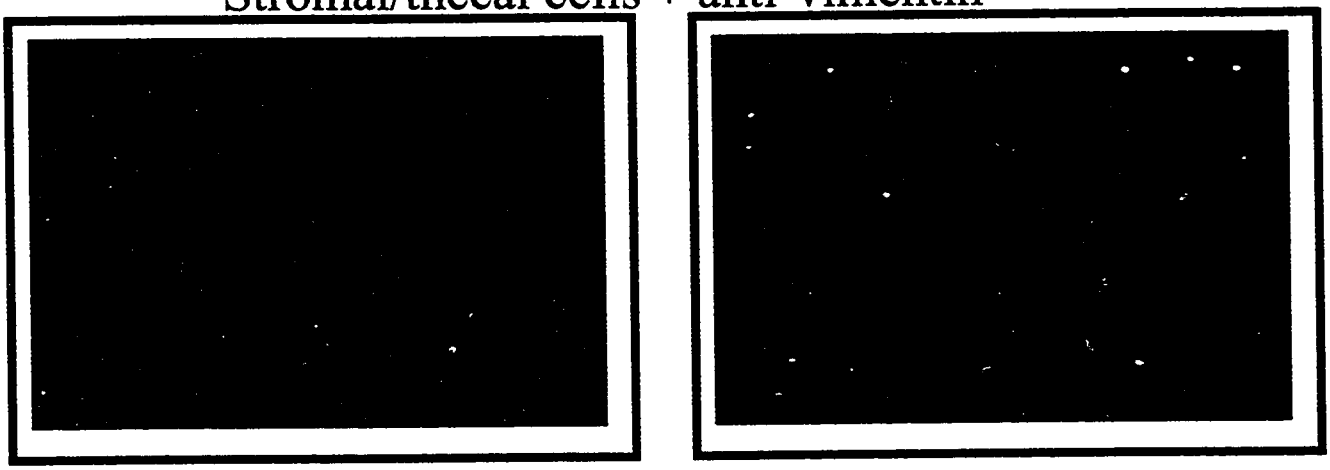
Stromal/thecal cells + no anti-vimentin

A



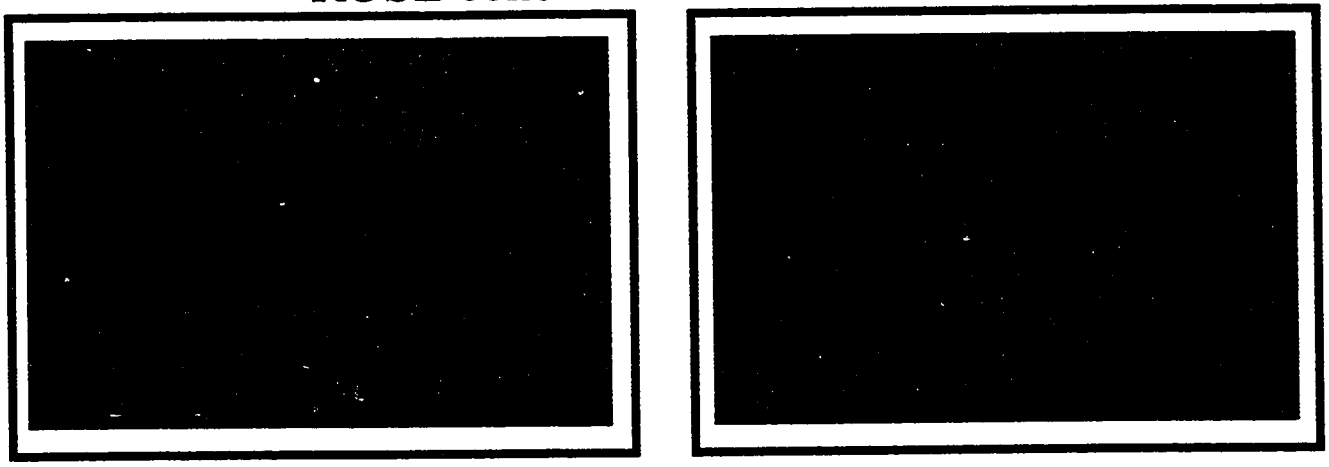
Stromal/thecal cells + anti-vimentin

B



ROSE cells + anti-vimentin

C



13. Effects of Growth Factors on ROSE 199 Cell Proliferation

The effects of three stromal/thecal-derived growth factors, TGF-β, TGF-α, and EGF, on ROSE 199 cell proliferation was assessed by culturing these cells in the presence or absence of various concentrations of each of the growth factors, in 10% (TGF-β and EGF) and 1% (TGF-α) FCS-containing medium for 48 hours. The results of these proliferation experiments are depicted in Figure 13. Significant changes in cell proliferation was observed only with TGF-β (ANOVA $p=0.0033$), at 1 ($p<0.05$), 10 ($p<0.01$), and 100 ng/ml ($p<0.01$), but not at 0.1 ng/ml ($p>0.05$) caused a greater than 40% decrease in cell proliferation compared to the control group (Figure 13A). These concentrations of EGF, on the other hand, had very little effect on the proliferation ROSE 199 (Figure 13B). To eliminate the possibility that FCS was masking the effects of EGF, and since both EGF and TGF-α act through the same receptor, the amount of FCS was lowered from 10% to 1% when treating the cells with TGF-α. This however still had very little effect on ROSE 199 cell proliferation (Figure 13C).

14. Effects of Stromal/Thecal-Derived Factors and Gonadotropins on Primary ROSE Cell Proliferation

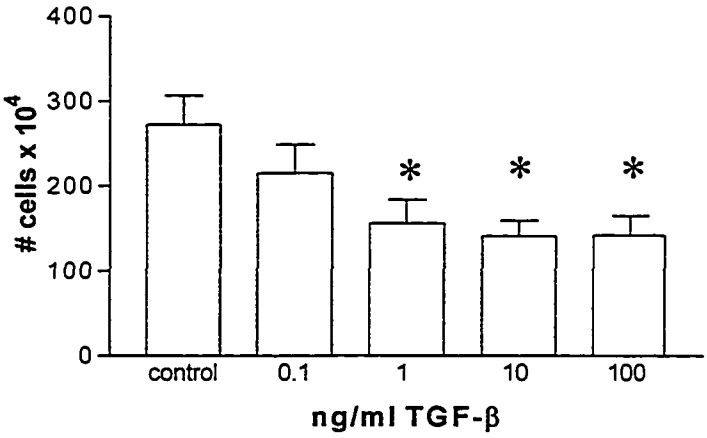
14.1 Effects of Growth Factors on Primary ROSE Proliferation

Recognizing the limitations in using an immortalized cell line such as ROSE 199 in these experiments, primary cultures of ROSE cells were also treated with the same three growth factors. In contrast with the results observed with the immortalized cell line,

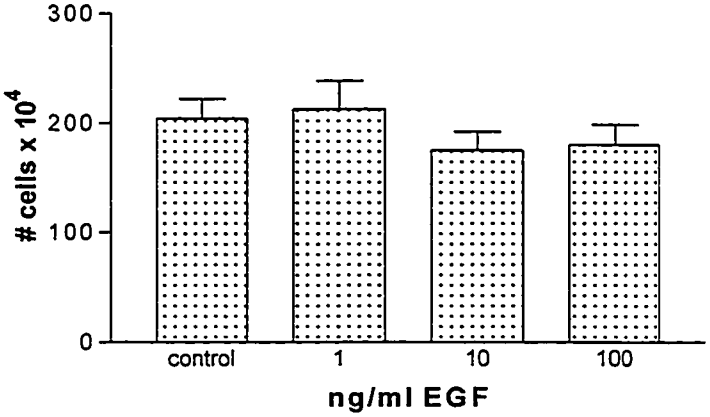
Figure 13: Effects of growth factors on ROSE 199 cell proliferation.

ROSE 199 cells were seeded at a density of $1.5x10^5$cells/100mm dish and cultured in 10% (TGF-β and EGF) or 1% (TGF-α) FCS-containing medium for 48 hours in 0.1, 1, 10 or 100 ng/ml TGF-β (A), and 1, 10, or 100 ng/ml EGF (B) or TGF-α (C). Proliferation was assessed by counting the number of cells with a haemocytometer. Each data point represents the mean± standard error of the mean (S.E.M.) of at least 3 experiments with duplicate treatments in each experiment. The TGF-α experiment was only performed once. Data were analysed by one-way ANOVA followed by Dunnett's Post Test. Bars indicated with an asterisk are significantly different from control ($p<0.05$).

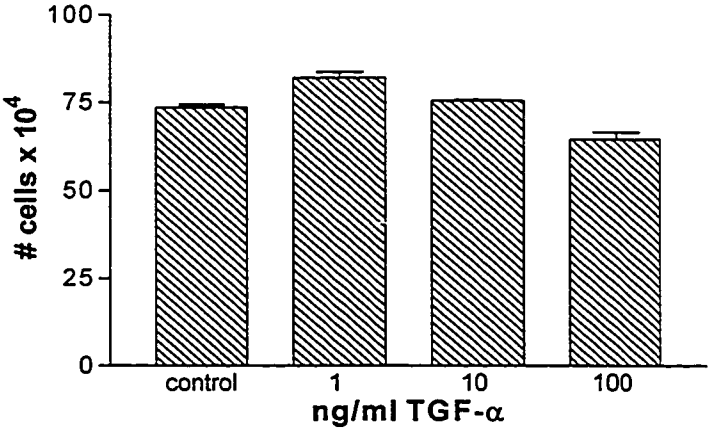
A


400
300
200
100
0
cells x 10^4
*
*
*
control
0.1
1
10
100
ng/ml TGF-β

B


300
200
100
0
cells x 10^4
control
1
10
100
ng/ml EGF

C


100
75
50
25
0
cells x 10^4
control
1
10
100
ng/ml TGF-α

treating primary ROSE cells with 10 ng/ml of any of the three growth factors significantly ($p<0.0001$ in all instances) altered the proliferation of these cells, as depicted in Figure 14. While TGF-β decreased primary ROSE cell proliferation by 70%, both TGF-α and EGF caused a twofold increase in the proliferation of these cells.

14.2 Effects of Gonadotropins on Primary ROSE Proliferation

In addition, ROSE cells were also grown in various concentrations of gonadotropic hormones, which are known to fluctuate at the time of ovulation. Although hCG increased the proliferation of ROSE cells by 1.7 and 1.8-fold, at 5 or 50 IU/ml respectively, these increases were not significant (Figure 15A). Treatment with various concentrations of FSH also had very little effect on the proliferation of ROSE cells (Figure 15B).

14.3 Effects of SCM on Primary ROSE Cell Proliferation.

In order to more closely mimic the in vivo situation, primary ROSE cells were treated with SCM which was generated by one of two methods, as described in section 10. As depicted in Figure 16 A and B, SCM generated by either method decreased the proliferation of ROSE cells at all time points investigated. When ROSE cells were directly treated with SCM (Figure 16A) their proliferation was decreased by 35 and 57% after 2 or 4 days in culture, respectively ($p<0.0001$ at 2 days).

To eliminate the potential effect of media depletion as might occur when SCM was generated by the first method, ROSE cells were subsequently treated with SCM

Figure 14: Effects of growth factors on primary ROSE cell proliferation

Primary ROSE cells were seeded at a density of $7.5x10^4$cells/60 mm dish and cultured for 4 days in 10 ng/ml of TGF-β, TGF-α or EGF. Proliferation was assessed by counting the number of cells with a haemocytometer. Each data point represents the mean±S.E.M of 3 experiments with 3 replicates per treatment in each experiment. Data were analysed by paired t-test. Those bars indicated with an asterisk are significantly different from control ($p<0.05$).

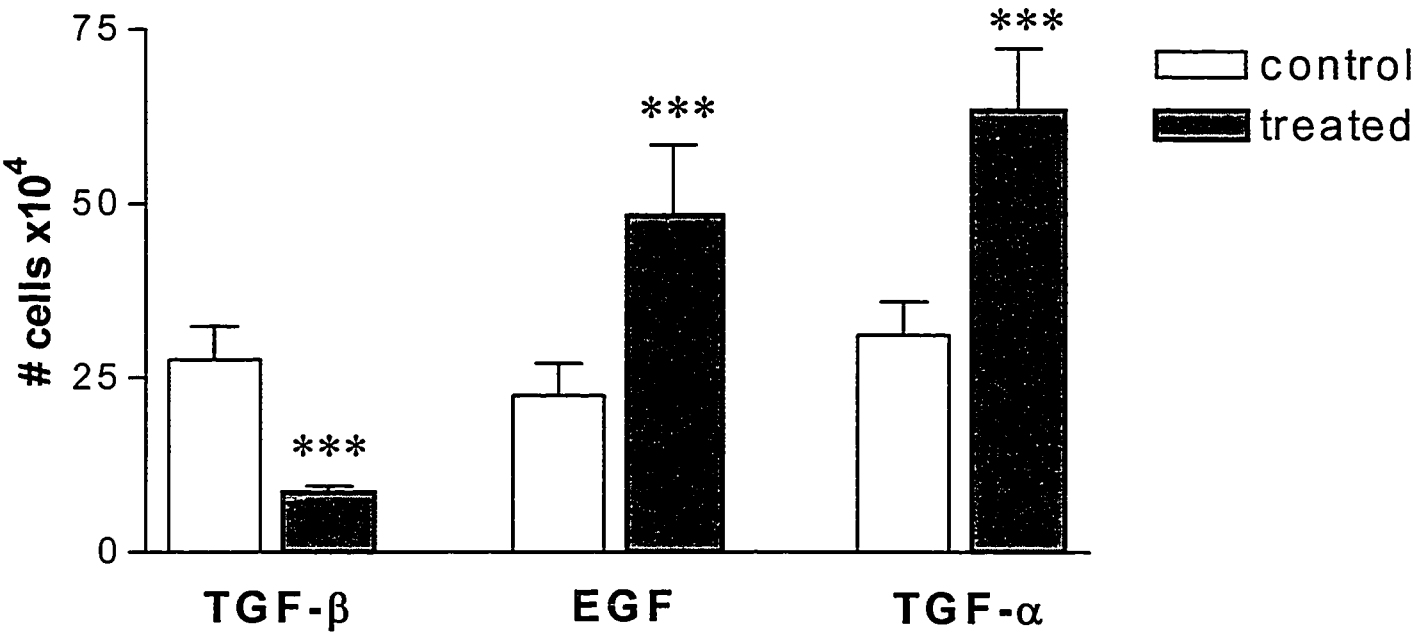
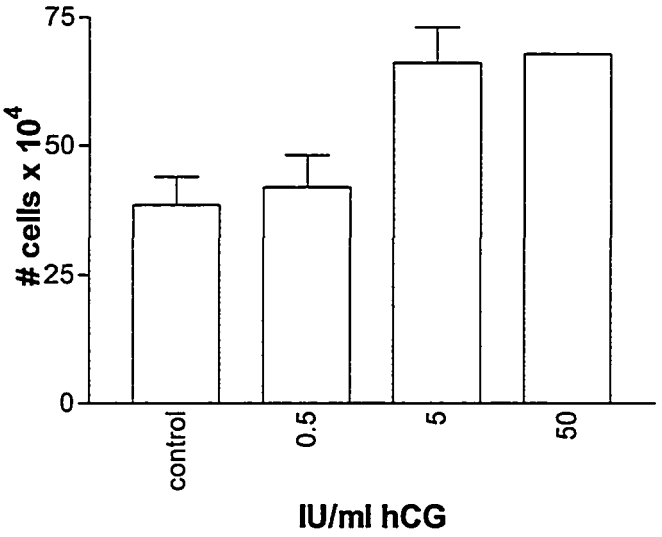

75
50
25
0
cells x10^4
***
***
***
TGF-β
EGF
TGF-α
control
treated

Figure 15: Effects of gonadotropins on primary ROSE cell proliferation

Primary ROSE cells were seeded at a density of $7.5x10^4$ cells/60 mm dish and cultured for 4 days in the absence or presence of 0.5, 5, or 50 IU/ml of hCG in A, and 10, 100, or 150 ng/ml of FSH in B. Proliferation was assessed by counting the number of cells with a haemocytometer. Each data point represents the mean±S.E.M of 3 experiments done in triplicate, except for 50 IU/ml which was done only once. Data were analysed by one-way ANOVA.

A


75
50
25
0
cells x 10^4
control
0.5
5
50
IU/ml hCG

B

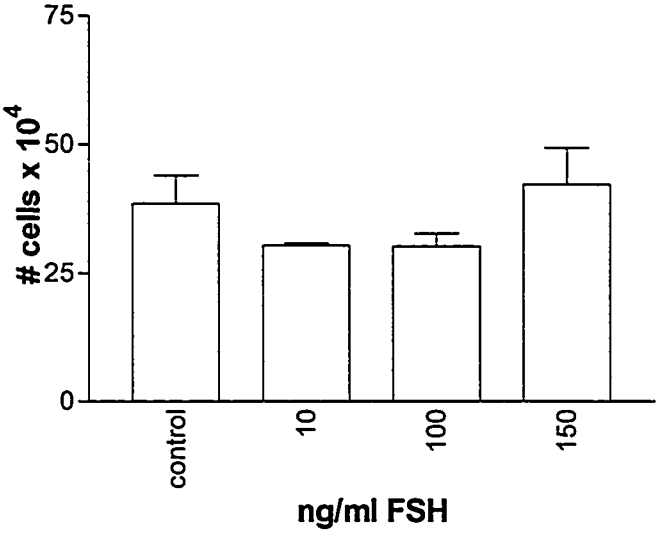
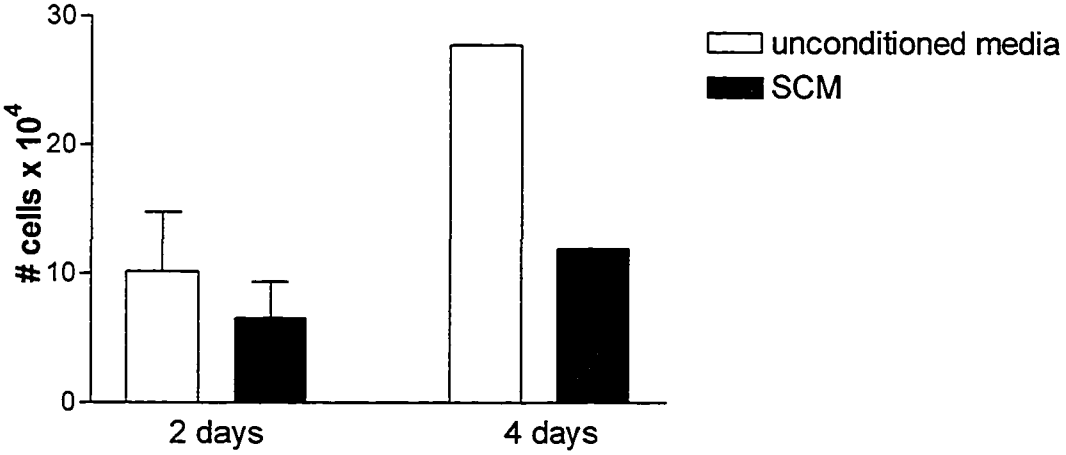

75
50
25
0
cells x 10^4
control
10
100
150
ng/ml FSH

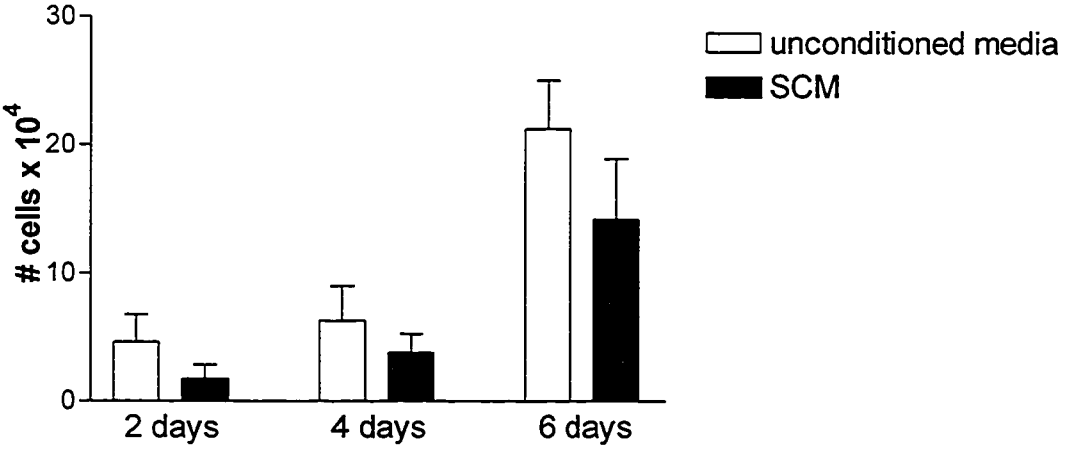
Figure 16: Effects of SCM on primary ROSE cell proliferation

Primary ROSE cells were seeded at a density of $5x10^4$ cells per 16 mm well and cultured for 2 or 4 days in SCM generated by method 1 (see section 10) in A, and at a density of $2.5x10^4$ cells/well for 2, 4 or 6 days in SCM generated by method 2 (see section 10), in B. Proliferation was assessed by counting the number of cells with a haemocytometer. Each data point represents the mean ±S.E.M. of 2 experiments at 2 days and 1 at 4 days in A, and 3-5 experiments in B, done in triplicate. Data were analysed by paired t-test in A and two-way ANOVA in B.

A


30
20
10
0
cells x 10^4
2 days
4 days
unconditioned media
SCM

B


30
20
10
0
cells x 10^4
2 days
4 days
6 days
unconditioned media
SCM

which was first concentrated using a method that would retain any stromal-secreted components with a MW greater than 3000, and then reconstituted in fresh media + 10% FCS (Figure 16B). SCM generated by this method inhibited the growth of ROSE cells by 60, 31 and 33% after being cultured for 2, 4 or 6 days, respectively. Although these decreases were not significant when analyzed by ANOVA ($p<0.0004$ for time; $p=0.1525$ for SCM; $p=0.7319$ for interaction), t-test performed on data at each time point indicated significant decreases ($p<0.0001$ at all time points).

14.4 Effects of hCG in Modifying the Regulation of Primary ROSE Cell Proliferation by SCM

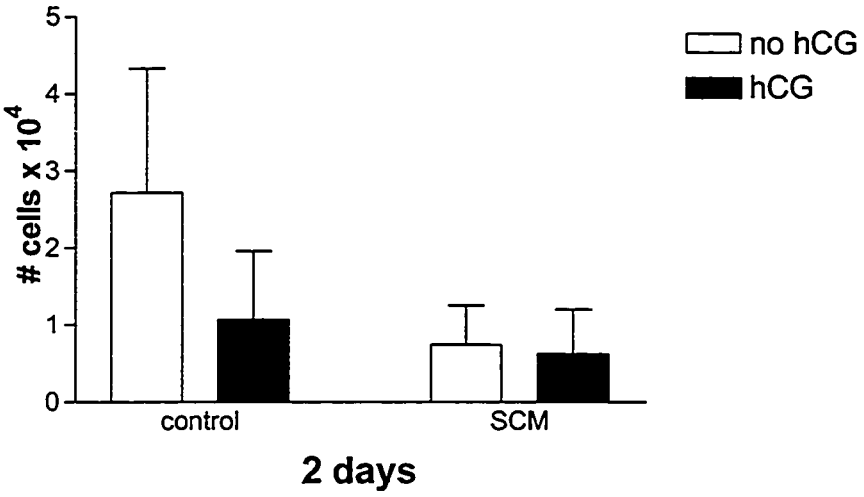
To investigate the effects of LH in modifying the OSE-stromal/thecal cell interactions, ROSE cells were also treated with SCM that was conditioned in the presence or absence of 200 ng/ml hCG (Figure 17). As observed in the previous experiment (Figure 16B), SCM decreased the proliferation of ROSE cells compared to unconditioned media at all time points investigated. Treating the stromal/thecal cells with hCG during SCM generation, did not seem to alter the effect of SCM on ROSE cell proliferation ($p<0.0001$ for time; $p= 0.082$ for SCM; $p=0.396$ for hCG; $p=0.05$ for interaction).

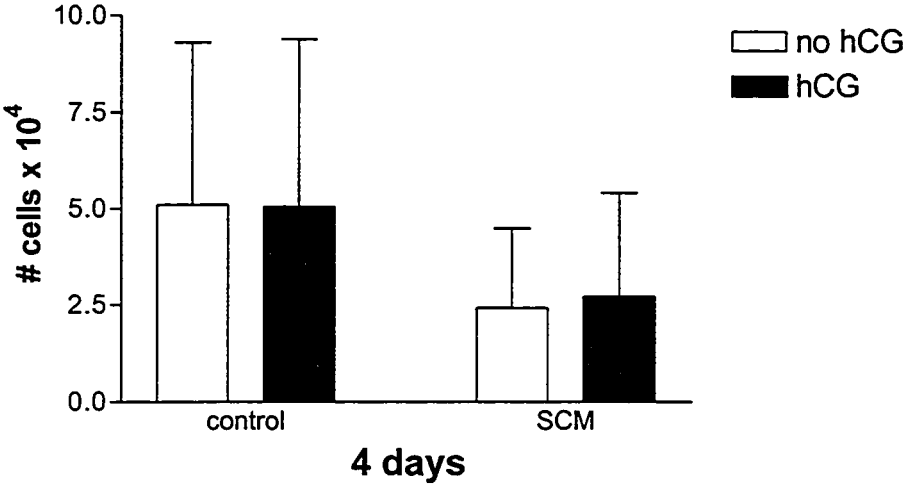
14.5 Effects of Blocking TGF-β on Cell Proliferation of ROSE Cultured in SCM.

In order to test the activity of the antibody depicted in Figure 18B, the neutralizing ability of the anti-TGF-β antibody in ROSE cell cultures was investigated (Figure 18A). Although treating ROSE cells with 400 ng/ml anti-TGF-β antibody alone did not affect ROSE cell proliferation, treating them with 10ng/ml TGF-β significantly decreased their

Figure 17: Effects of hCG in modifying the regulation of primary ROSE cell proliferation by SCM

Primary ROSE cells were seeded at a density of $2.5x10^4$ cells per 16 mm well and cultured for 2 (A), 4 (B) or 6 (C) days in SCM generated by method 2 (see section 10), where stromal/thecal cells were cultured in the presence of absence of 200 ng/ml hCG. Proliferation was assessed by counting the number of cells with a haemocytometer. Each data point represents the mean ±S.E.M of two experiments in A and three in B and C, done in duplicate or triplicate. Data were analysed by three-way ANOVA.


5
4
3
2
1
0
cells x 10^4
no hCG
hCG
control
SCM
2 days


10.0
7.5
5.0
2.5
0.0
cells x 10^4
no hCG
hCG
control
SCM
4 days

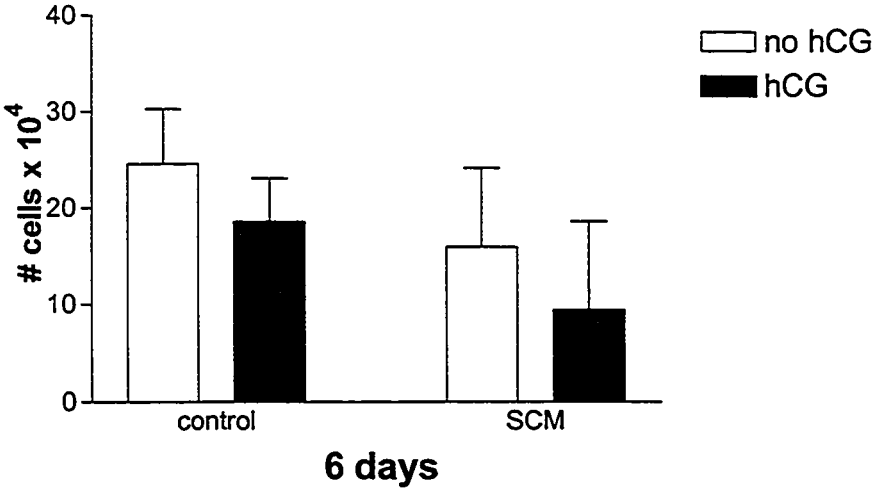
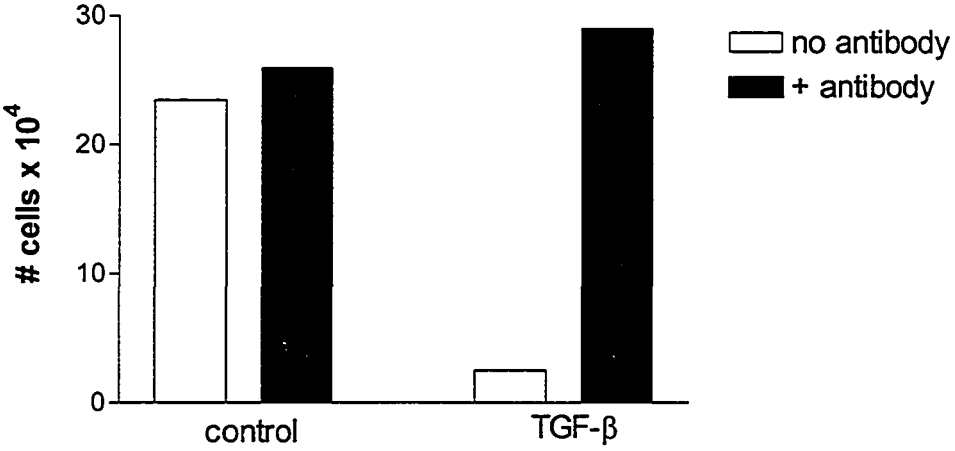

40
30
20
10
0
cells x 10^4
no hCG
hCG
control
SCM
6 days

Figure 18: Effects of blocking TGF-β in SCM on ROSE cell proliferation

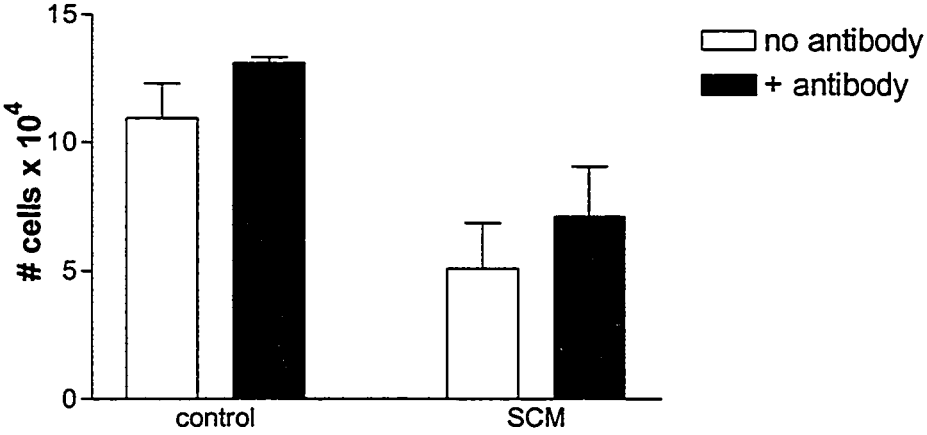
In Figure A, the ability of the TGF-β antibody to neutralize TGF-β activity in ROSE cell cultures was tested. Primary ROSE cells were seeded at a density of $7.5x10^4$ cells/60 mm dish and cultured for 4 days in 10 ng/ml of TGF-β, 400 ng/ml of TGF-β antibody, or in the presence of both 10 ng/ml TGF-β and 400 ng/ml TGF-β antibody. Proliferation was assessed by counting the number of cells with a haemocytometer. Each data point represents the mean of 1 experiment done in duplicate.

In Figure B, the effect on blocking TGF-β in SCM was investigated. Primary ROSE cells were seeded at a density of $2.5x10^4$ cells per 16 mm wells and cultured for 4 days in SCM generated by method 2 (see section 10), ±20 ng/ml anti-TGF-β antibody. Proliferation was assessed by counting the number of cells with a haemocytometer. Each data point represents the mean ±S.E.M of two experiments done in triplicate. Data were analysed by two-way ANOVA.

A


30
20
10
0
cells x 10^4
control
TGF-β
no antibody
+ antibody

B


15
10
5
0
cells x 10^4
control
SCM
no antibody
+ antibody

proliferation by 89%, as shown in previous experiments (see Figure 14 for previous experiments). When ROSE cells were treated with 400 ng/ml anti-TGF-β antibody in addition to the 10 ng/ml TGF-β, the anti-TGF-β antibody was able to completely neutralize the growth inhibition effect of 10 ng/ml TGF-β ($p=1.0$ for TGF-β, anti-TGF-β antibody and for interaction.

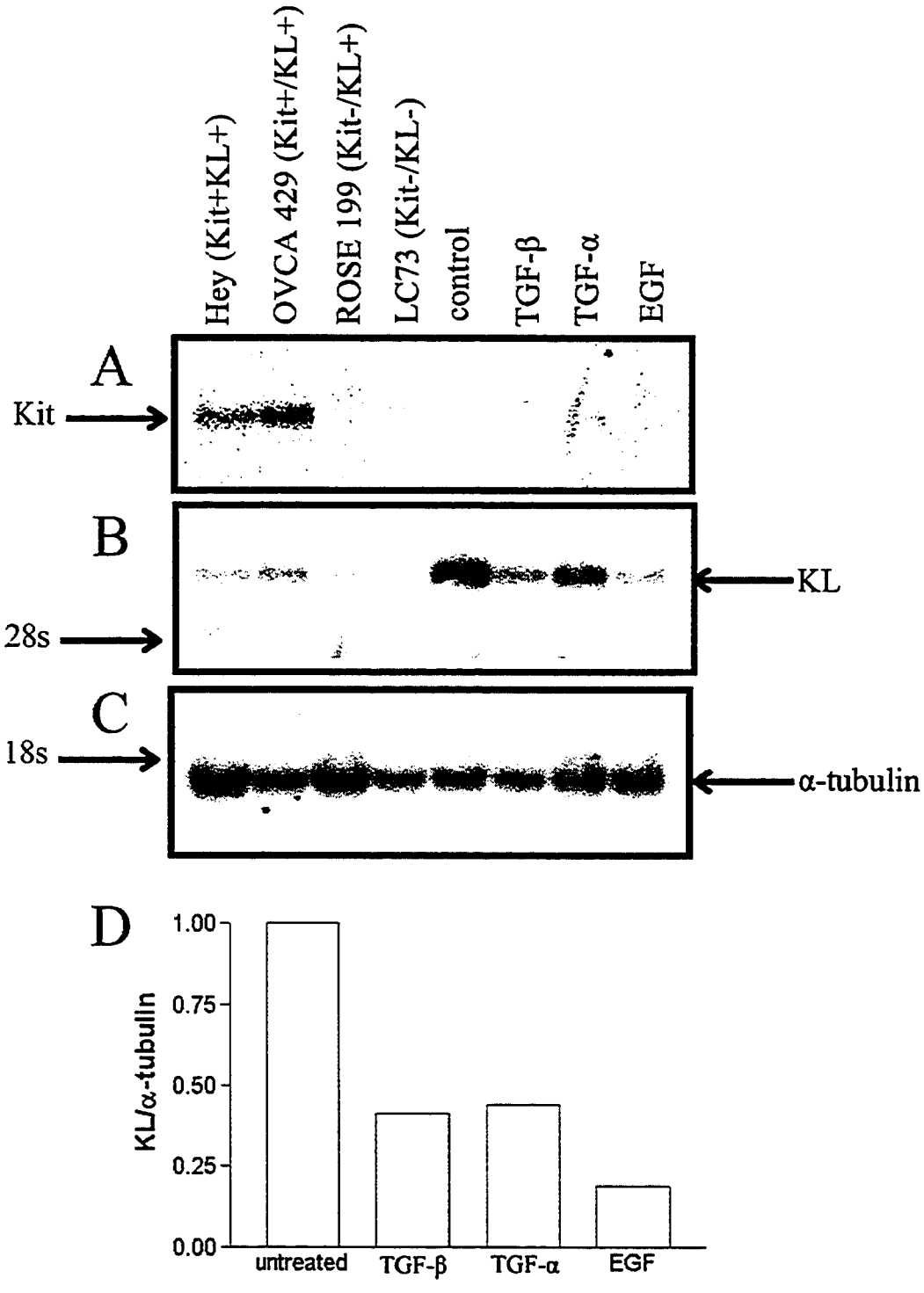
As depicted in Figure 18B, an attempt was made to identify TGF-β as a potential mediator of the decreased proliferation observed in ROSE cells cultured in SCM (see Figure 16). As expected, culturing ROSE cells in SCM decreased their proliferation by 53% compared to control. Treating ROSE cells or ROSE cells cultured in SCM with 20 ng/ml anti-TGF-β Ab did not alter proliferation from their respective controls.

14.6 Effects of Stromal/Thecal-Derived Factors and Gonadotropins on c-*kit* and KL mRNA Expression in Primary ROSE Cells

The effects of stromal/thecal-derived factors on c-*kit* and KL mRNA expression in primary ROSE cells were investigated by Northern blot analysis (Figure 19) and RT-PCR (Figures 20 and 21). As depicted in Figure 19A, c-*kit* mRNA was not detected in untreated (control) primary ROSE cells, and its expression was not induced when ROSE cells were cultured for 4 days in 10 ng/ml TGF-β, TGF-α, or EGF. KL mRNA on the other hand was detected in both untreated and growth factor-treated primary ROSE cells (Figure 19B). However, comparing KL and α-tubulin band intensity revealed that KL

Figure 19: Northern analysis of c-*kit* and KL mRNA in primary ROSE cells cultures in the presence of growth factors

Northern blot analysis was performed on 15 μg of total RNA from primary cultures of ROSE cells cultured for 4 days in the absence or presence of 10 ng/ml TGF-β, TGF-α, or EGF. The blot was sequentially probed with ^{32}P-labelled cDNA probes for the human KIT receptor (A), human KL (B), and α-tubulin (C). Hey and OVCA 429 cells (ovarian carcinoma) served as positive controls for c-*kit* while ROSE 199 and LC73 (lung cancer) served as negative controls (A). Hey, OVCA 429 and ROSE 199 cells served as positive controls for KL while LC73 cells served as negative control (B). Band intensity was estimated using ImageQuaNT software and the ratios of KL/α-tubulin values were standardised to control levels, arbitrarily set to 1.0 (D). This blot is representative of two experiments with similar results.


Hey (Kit+KL+)
OVCA 429 (Kit+/KL+)
ROSE 199 (Kit-/KL+)
LC73 (Kit-/KL-)
control
TGF-β
TGF-α
EGF
A
Kit
B
KL
28s
C
18s
α-tubulin
D
KL/α-tubulin
1.00
0.75
0.50
0.25
0.00
untreated
TGF-β
TGF-α
EGF

mRNA abundance was decreased by more than half when the cells were treated with TGF-β, TGF-α, or EGF (Figure 19D).

In Figure 20 the relative proportion of transcripts encoding membrane-bound versus soluble forms of KL was investigated by RT-PCR in untreated and growth factor-treated ROSE cells. The soluble form (287 bp transcript) was much more predominant than the membrane-bound form (203 bp transcript)and the growth factors had no effect on the relative proportions of the two transcripts.

Although c-*kit* mRNA was not detected in ROSE cells by Northern blot analysis (Figure 19), RT-PCR for c-*kit* revealed some c-*kit* mRNA expression in both untreated and growth factor- or gonadotropin-treated ROSE cells (Figure 21). Although this was not a quantitative PCR, all treatments, and especially TGF-β and hCG seemed to decrease c-kit *mRNA* expression compared to control.

14.7 Effects of SCM on c-*kit* mRNA Expression in Primary ROSE Cells

The effects of SCM on c-*kit* mRNA expression in primary ROSE cells were investigated by RT-PCR (Figure 22). c-*kit* mRNA was not detected in control groups of three independent experiments. However, it was detected in all treatment groups of experiment 2 (ROSE cells cultured in control medium+hCG, SCM and SCM conditioned in the presence of hCG), but not in any of the treatment groups of the other two experiments. As expected, c-*kit* transcripts were also found in total ovary RNA and stromal/thecal cell RNA.

Figure 20: RT-PCR for KL in ROSE cells cultured in presence of growth factors

Reverse transcription-polymerase chain reaction was performed on 1.5 μg total RNA from primary ROSE cells cultured in the absence (control) or presence of 10 ng/ml of TGF-β, TGF-α, or EGF. KL primers spanning the alternatively spliced exon of KL mRNA were used to amplify transcripts for membrane-bound KL-2 (203 bp) and soluble KL-1 (287 bp) transcripts. ROSE 199 and MC/9 served as positive and negative controls, respectively. RNA was substituted with water prior to RT-PCR to serve as a negative control. The RT-PCR products were resolved on a 2% agarose gel run at 100 V for 1 hour and then stained with ethidium bromide. These results are representative of two experiments.

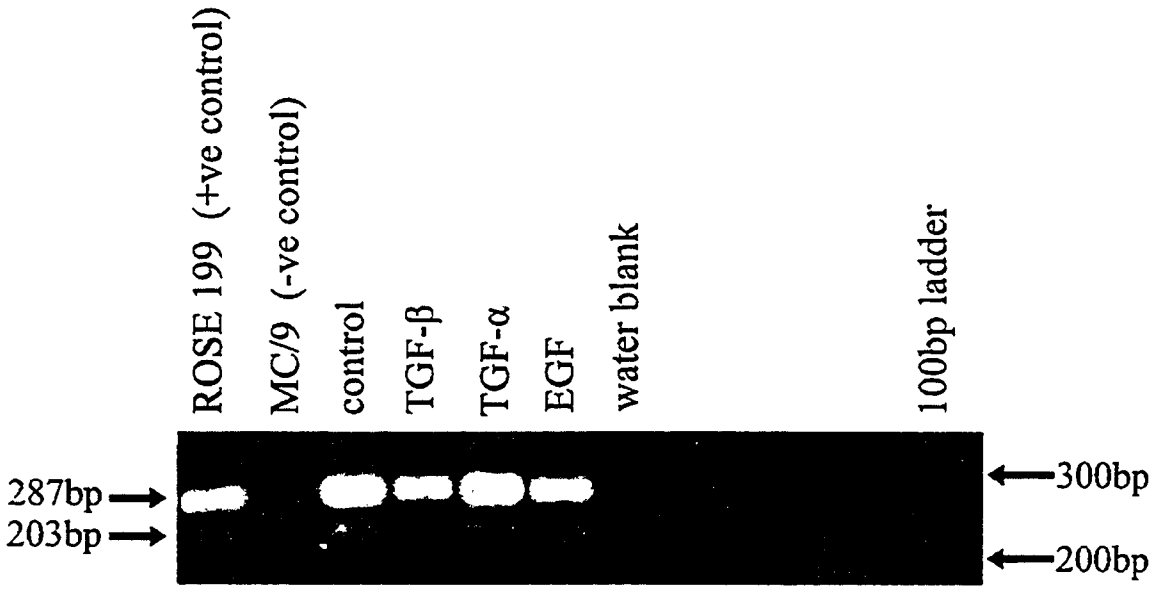

ROSE 199 (+ve control)
MC/9 (-ve control)
control
TGF-β
TGF-α
EGF
water blank
100bp ladder
287bp
203bp
300bp
200bp

Figure 21: RT-PCR for c-*kit* in ROSE cells cultured in the presence of growth factors and gonadotropins

RT-PCR was performed on 1.5 μg total RNA from primary ROSE cells cultured in the absence (control) or presence of 10 ng/ml of TGF-β, TGF-α, or EGF, and hCG or FSH (pooled samples, 0.5, 5, 50 IU/ml hCG and 10, 100, 150 ng/ml FSH). The nucleotide positions of the 5' and 3' c-*KIT* primers were bp 1326-1346 and bp 1874-1898, respectively, yielding a 572 bp PCR product. Rat ovary and 3T3 cells served as positive

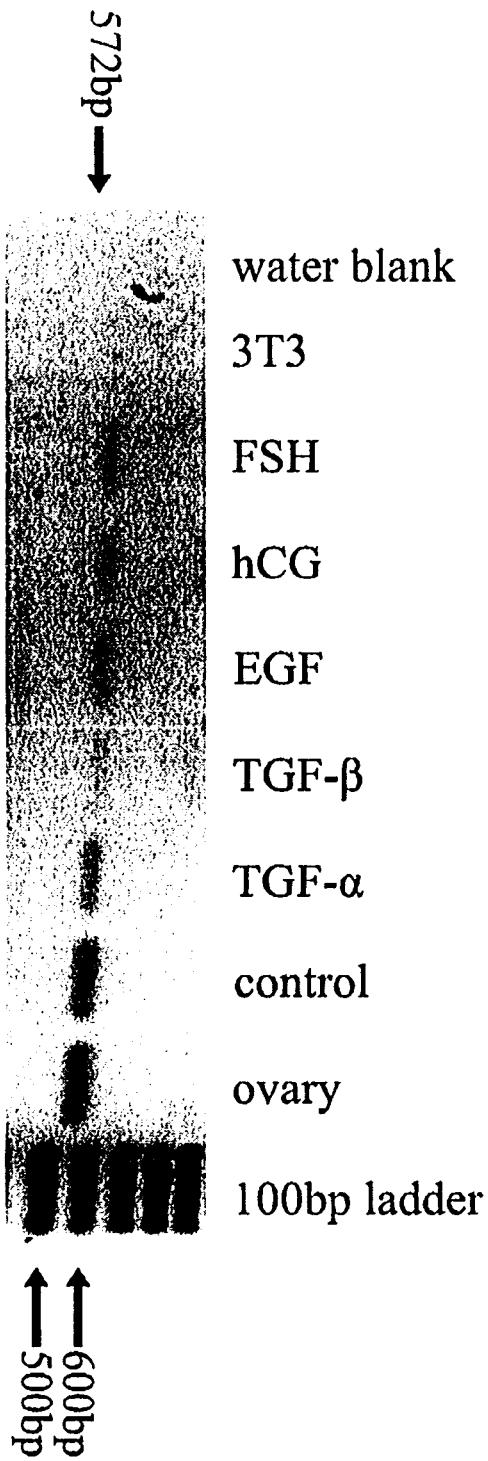

572bp
water blank
3T3
FSH
hCG
EGF
TGF-β
TGF-α
control
ovary
100bp ladder
500bp
600bp

Figure 22: RT-PCR for c-*kit* in ROSE cells cultured in SCM ±hCG

RT-PCR was performed on 1.5 μg total RNA from primary ROSE cells cultured in the absence (control and control hCG) or presence of SCM ±hCG. The nucleotide positions of the 5' and 3' c-*KIT* primers were bp 1326-1346 and bp 1874-1898, respectively, yielding a 572 bp PCR product. Rat ovary and 3T3 cells served as positive and negative controls, respectively. RNA was substituted with water prior to RT-PCR to serve as a negative control. The RT-PCR products were resolved on a 1% agarose gel run at 100 V for 1 hour and then stained with ethidium bromide. These results are representative of three experiments.

572bp
water blank
3T3
stromal/thecal cells
SCM + hCG 3
SCM + hCG 2
SCM + hCG 1
control hCG 3
control hCG 2
control hCG 1
SCM 3
SCM 2
SCM 1
control 3
control 2
control 1
ovary
100bp ladder
600bp
500bp

DISCUSSION

This project was designed to address the possible involvement of ovarian stromal/thecal cells in the regulation of OSE cell growth and Kit and KL expression. Such interactions are probably involved in normal OSE-stromal/thecal cell activities as well as in interactions occurring within inclusion cysts and leading to ovarian tumour formation. Although TGF-β inhibited growth of both ROSE 199 and primary ROSE cells, TGF-α, EGF and hCG stimulated the growth of primary ROSE but not immortalized ROSE cells. FSH had no effect on the proliferation of either cell type. SCM also had a growth inhibitory effect on ROSE cells, but adding TGFβ neutralizing antibody did not eliminate this effect, suggesting the involvement of other mediators. All three exogenously added growth factors also decreased KL mRNA expression in ROSE cells, but none of the factors investigated were found to induce expression of Kit in the primary ROSE cells, by Northern analysis.

The purity of the primary ROSE and stromal/thecal cell preparations was confirmed by their characteristic morphological features as well as keratin and vimentin expression. 100% of all primary ROSE cell cultures exhibited a typical epithelioid morphology and were arranged in an orderly cobblestone pattern suggesting the absence of contamination by other ovarian cell types (Adams and Auersperg, 1981). The high proportion (67-97%) of ROSE cells positive for keratin also confirmed the purity of the cultures established. Greater keratin staining might have been achieved by staining freshly isolated ROSE cells since the proportion of keratin-positive cells has been shown to decrease with increasing passage in cultured hOSE (Auersperg *et al.*, 1994). Since OSE cells are known to express both epithelial and mesenchymal characteristics, the high

frequency of vimentin staining reported here in ROSE cells and by Auersperg *et al.* (1994) and Hornby *et al.* (1992) in hOSE and in immortalized ROSE cell lines respectively, was not unexpected.

Both the high proportion of vimentin staining along with complete lack of keratin staining, and characteristic fibroblastic morphology of the primary cultures of stromal/thecal cells confirmed the purity of these cultures and lack of contamination by OSE cells.

TGF-β had the expected effect of substantially inhibiting the proliferation of both ROSE 199 and primary ROSE cells. As both TGF-β (Berchuck *et al.*, 1992b; Nilsson *et al.*, 2001), and components of the TGF-β receptor system (Jindal *et al.*, 1995; Bartlett *et al.*, 1997; Nilsson *et al.*, 2001), have been shown to be expressed in normal and transformed OSE cells , this effect of TGF-β on OSE may result from an autocrine action in vivo. Alternatively, paracrine interactions involving thecal cells are also a possibility as thecal cells have been shown to produce TGF-β (Skinner *et al.*, 1987; Chegini and Flanders, 1992; Mulheron *et al.*, 1991). The ability of transformed OSE to escape this inhibitory effect could contribute to ovarian cancer initiation or progression.

As for rat and other species investigated (Stromberg *et al.*, 1992; Godwin *et al.*, 1992; Siemens and Auersperg, 1988; Rodriguez *et al.*, 1991; Crew *et al.*, 1992), EGF and TGF-α greatly increased the proliferation of primary ROSE cells. However, neither of these growth factors had an effect on the proliferation of ROSE 199, suggesting limitations in using an immortalized cell line and stressing the importance of using primary cultures. Possible explanations for this lack of responsiveness to EGF and TGF-α by the immortalized cell line include maximal stimulation by growth factors from the

culture media or by an autocrine loop, or loss of EGF receptor expression. Although ROSE 199 cells were cultured in media supplemented with 10% FCS when treated with EGF, an attempt was made to eliminate the possibility of maximal stimulation by growth factors from the culture media by culturing the cells in 1% FCS when treated with TGF-α. This attempt however did not improve the response of the cells. Growing the cells in serum free-media might have changed their response. An EGF/TGF-α autocrine loop remains a possibility as normal and transformed OSE cells have been reported to express both the ligand and the receptor (Stromberg *et al.*, 1992; Berchuck *et al.*, 1991; Rodriguez *et al.*, 1991). The loss of EGF receptor has also been reported in epithelial ovarian cancer cells (Berchuck *et al.*, 1991); however, neither the co-expression of EGF/TGF-α and the EGF receptor, nor the loss of EGF receptor has been investigated in the ROSE 199 cell line. Another possible argument for the lack of responsiveness observed is an insufficient concentration of the growth factors added, which ranged from 1 to 100ng/ml in this study. This is very unlikely, however, since most groups have reported stimulatory effects at concentrations of 20-30ng/ml (Godwin *et al.*, 1992; Siemens and Auersperg, 1988; Rodriguez *et al.*, 1991). Like TGF-β, the actions of EGF/TGF-α on OSE cells are often interpreted as autocrine actions. However, these stromal/thecal-derived growth factors could also play a role in wound repair via paracrine interactions, and interactions gone awry might lead to growth stimulation and inclusion cyst or tumour formation.

Although gonadotropic hormones are not stromal/thecal-derived factors, primary cultures of ROSE cells were treated with various concentrations of hCG and FSH to mimic the in vivo environment at the time of ovulation and investigate the possible direct

effect these hormones might have on OSE cells. In agreement with reports on OSE cells in other species (Konishi *et al.*, 1999; Osterholzer *et al.*, 1985b; Parrott *et al.*, 2001; Syed *et al.*, 2001), hCG increased the proliferation of ROSE cells. Repeated exposure to LH as would occur in incessant ovulation or exposure to greater concentrations of LH within inclusion cyst could contribute to ovarian cancer by increasing growth and/or transformation of OSE cells.

The effects of FSH on OSE cell proliferation remain controversial, as both inhibitory and stimulatory effects have been reported. Insufficient FSH concentrations might account for the lack of responsiveness observed in the present study, as growth stimulation has been reported by Osterholzer *et al.* (1985b) where rabbit OSE cells were treated with 1 μg/ml of FSH in serum-free media, compared to a maximum of 150 ng/ml in 10% FCS used in the present study. However, such elevated concentrations of FSH within the ovary are very unlikely as others have reported maximal concentrations of FSH in human follicular fluid to be 5.4 ng/ml (Suchanek *et al.*, 1994). Alternatively, the lack of response could be attributable to the lack or loss of FSH receptor in rat OSE, or simply to species differences.

To mimic more closely the in vivo situation, SCM was generated to replace the above exogenous stromal/thecal factors. Both fresh and reconstituted SCM decreased the proliferation of ROSE cells, indicating that the process of concentrating and reconstituting the SCM did not remove its inhibitory action, that the MW of the factor mediating this effect is greater that 3 kD, and further supporting the hypothesis that stromal/thecal secrete factors able to regulate OSE cell growth. Karlan *et al.* (1995a) have also reported that SCM from human ovarian stromal cells inhibited the growth of

normal and epithelial ovarian cancer cells (Karlan *et al.*, 1995a). Conversely, Vigne *et al.* (1994) have reported a small increase in the proliferation of bovine OSE cells treated with concentrated serum-free stromal-conditioned medium (Vigne *et al.*, 1994). In both Karlan's and the present study, the SCM was concentrated to 10X to remove cellular debris and metabolic waste, but was reconstituted to 1X prior to treating the OSE cells. Vigne *et al.* on the other hand not only concentrated the SCM 100-fold but used it undiluted to treat the cells. This difference in methodology might account for the discrepancy between this second report and that of Karlan *et al.* (1995a) and of the present project.

To investigate the involvement of the pre-ovulatory LH surge in modifying the OSE-stromal/thecal cell interactions, SCM was also generated by stromal/thecal cells cultured in the presence of hCG. Conditioning the stromal media in the presence of hCG did not alter its effect on ROSE cell proliferation. This lack of response might be accounted for by a resulting balance between the actions of these and other stromal/thecal secreted factors on OSE cells. Alternatively, the lack of response could come from the stromal/thecal cells, as Magoffin and Erickson (1981) have reported the inhibition of stromal/thecal cell response to hCG by estrogen, and the stromal/thecal cells used to establish the primary cultures for this project were collected from estrogen-primed animals.

In an attempt to identify the mediator of the growth inhibitory effect of SCM on ROSE cells, a TGF-β neutralizing antibody was added to the SCM. Under the conditions used, the antibody did not eliminate the growth inhibitory effect, indicating that TGF-β is not or not the only mediator of the SCM inhibitory effect. This experiment could be

further pursued by performing TGF-β antibody dose responses and adding the antibody to the SCM prior to treating the cells, to allow sufficient time for the neutralization of TGF-β to take place. Using a more biochemical approach, Karlan *et al.* (1995a) have attempted to characterize the "ovarian inhibitory substance". Their analyses suggested a small heat-stable, non-heparin-binding, water-soluble factor with a molecular weight between 1-3 kD, eliminating TGF-β (25 kD) as a possible candidate.

Kit has generally not been found to be expressed by normal OSE cells (Inoue *et al.*, 1994; Ismail *et al.*, 1999; Tonary *et al.*, 2000). One group has however reported Kit expression in both normal and transformed OSE (Parrott *et al.*, 2000b). The present project was designed and performed before the publication of this last finding, and the rational was therefore based on the absence of Kit expression in normal OSE. Kit expression has however been reported in ovarian cancer cells and tumours, and in OSE cells of inclusion cysts (Natali *et al.*, 1992c; Inoue *et al.*, 1994; Arber *et al.*, 1998; Tonary *et al.*, 2000). Investigating whether stromal/thecal-derived factors, gonadotropins and/or SCM could induce Kit expression in OSE was therefore one of the objectives of this project. Accordingly, Kit mRNA was not detected by Northern blot analysis in untreated primary ROSE cells, and although its expression was not induced by the presence of the three growth factors used in this study, other stromal/thecal secreted factors might have this ability. The ability of gonadotropins and SCM to induce Kit expression in ROSE was not investigated by Northern blot during this project due to the limited quantity of cells from which the RNA was extracted. Kit transcripts were however found by RT-PCR in controls, growth factor- and gonadotropin-treated ROSE cells. The detection of Kit transcripts by RT-PCR but not Northern blot suggests very low levels of c-Kit

transcription in these cells. The biological significance of these transcripts might better be evaluated by quantitative PCR and more importantly by immunohistochemistry or Western blotting. Although the RT-PCR performed during this project were not quantitative, the observed decrease in c-Kit transcription by TGF-β, hCG, and FSH is in agreement with the findings that TGF-β inhibits expression of Kit in haematopoietic progenitor cells (Dubois *et al.*, 1994; Heinrich *et al.*, 1995; Sansilvestri *et al.*, 1995), acute myelogenous leukemia blast cell (De Vos *et al.*, 1993) and colorectal carcinoma mucosa cells (Bellone *et al.*, 1997) and that both FSH and LH/hCG have been shown to decrease Kit expression in theca cells (Motro and Bernstein, 1993) and oocytes (Horie *et al.*, 1991) of mouse ovaries.

Contrary to the above Kit RT-PCR results, c-*kit* mRNA was not detected in control groups of three independent SCM experiments. However, it was detected in all treatment groups of experiment 2 (ROSE cells cultured in control medium+hCG, SCM and SCM conditioned in the presence of hCG), but not in any of the treatment groups of the other two experiments. The biological significance of these Kit transcripts remains to be determined as for the above experiments. The discrepancy between the presence and absence of Kit transcripts in the controls of the previous experiments and the controls of these three SCM experiments respectively might be due to the fact that different ROSE cell preparations were used, or to differences in the number of passages or confluency at the time when the cells were collected for RNA extraction. This explanation cannot be verified at this time since many of the RNA samples extracted from cells treated with the growth factors or gonadotropins were pooled from different experiments. The effect of passage number or confluency on Kit expression in cultured ROSE cells could however

very easily be investigated. Accordingly, a colleague in the lab has observed that the level of Kit expression in ovarian cancer cells is influenced by cell density, such that Kit expression is high at low cell density and low in confluent cells (Manuscript in preparation). The discrepancy between the results of SCM experiment 2 and the other two experiments is more difficult to explain. However, differences in the number of passages or confluency of the ROSE cells from which the experiments were set up might also account for a difference in the ability of the cells to express Kit transcripts under the specific conditions. Repeating these experiments in parallel, that is using the same preparations of ROSE cells to establish multiple experiments would eliminate some of these differences. The effects of repeated subculturing/repeated ovulation and/or overcrowding have been noted to induce transformation in OSE cells, leading to the acquirement of features including loss of contact inhibition (Adams and Auersperg, 1985; Godwin *et al.*, 1992), the capacity for substrate-independent growth and cytogenic abnormalities (Godwin *et al.*, 1992; Testa *et al.*, 1994), tumorigenicity (Abdollahi *et al.*, 1997; Godwin *et al.*, 1992) and differences in gene expression, (Schildkraut *et al.*, 1997; Abdollahi *et al.*, 1997).

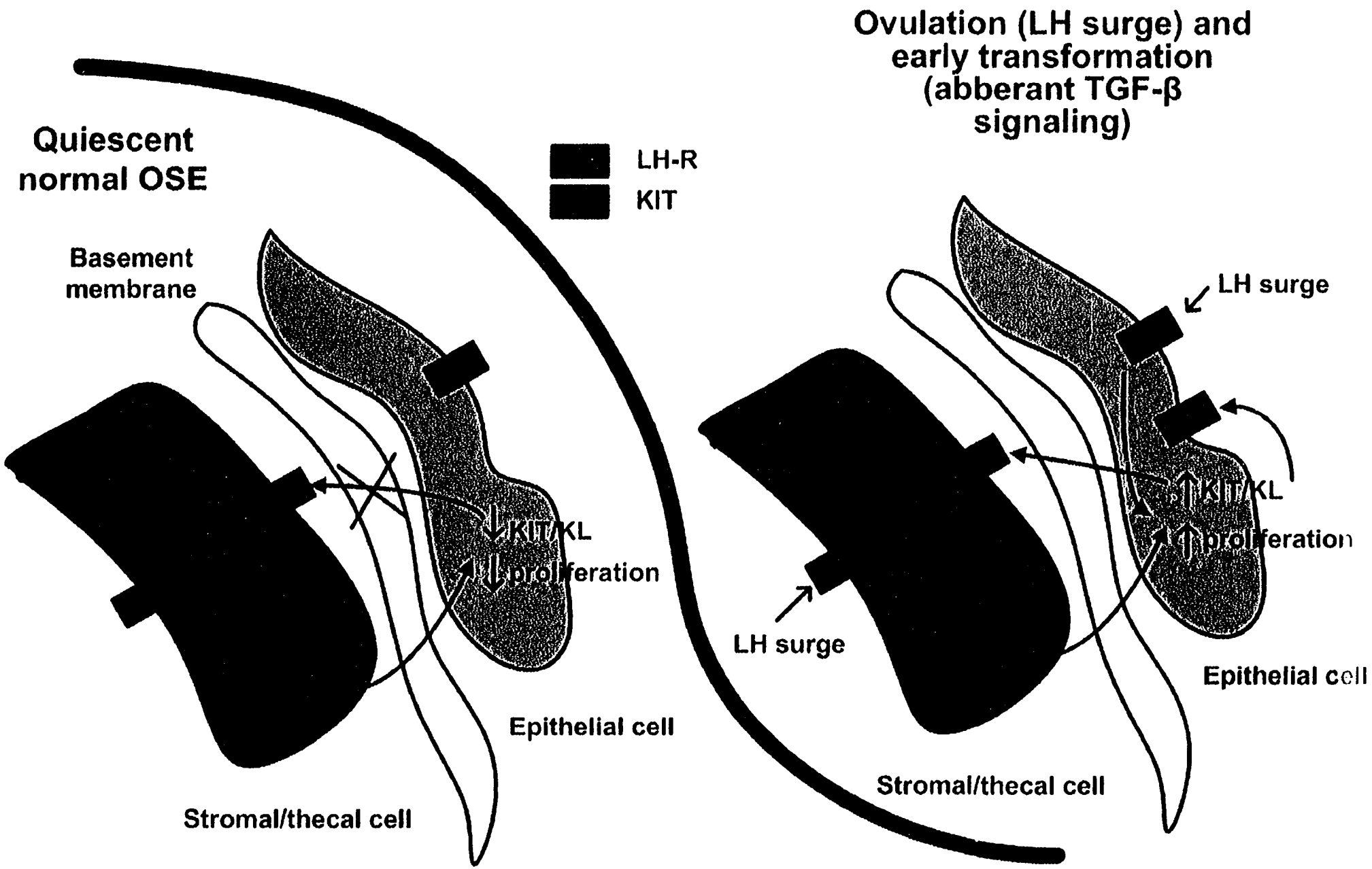
KL mRNA, on the other hand, was expressed in both untreated and growth factor-treated primary ROSE cells. TGF-β, TGF-α, and EGF all decreased its expression by more than half. Although TGF-β has previously been reported to inhibit expression of KL (Heinrich *et al.*, 1995; Ismail *et al.*, 1999), this is the first report of modulation of KL expression by TGF-α and EGF. Nilsson *et al.* (2001) have recently reported the opposite effect of TGF-β on KL expression in bovine OSE. Species-specific differences may account for the difference between these reports. As both OSE and stromal/thecal cells

produce these growth factors, the regulation of KL by these factors could result from either autocrine or paracrine interactions to regulate the growth of normally Kit expressing stromal/thecal cells or of transformed OSE expressing Kit. KL has been shown to have a stimulatory effect on bovine thecal cell growth and androgen production (Parrott and Skinner, 1997). The downregulation of KL in OSE might therefore lead to a decrease in these activities in thecal cells, which would inevitably impact on OSE cell activity.

ROSE cells have been shown to predominantly produce the soluble form of KL (Ismail *et al.*, 1999). Accordingly, soluble KL was the primary product of ROSE cells in basal conditions, and none of the growth factors investigated here altered the proportion of soluble to membrane-bound KL in these cells. These observations further suggest that the production of soluble KL by OSE cells could regulate activities of the underlying Kit expressing stromal/thecal cells. The regulation of KL expression in ROSE by gonadotropins and SCM was not investigated in this study due to the limited quantity of cells from which the RNA was extracted.

The regulation of growth and Kit and KL expression in ROSE cells by stromal/thecal-derived factors clearly suggests that the paracrine interactions being investigated will involve a cascade of growth factors produced by either cell type and be regulated during the ovulatory cycle. Based on the findings reported here and in the literature, a model is proposed (Figure 23) for OSE-stromal/thecal cell interactions under normal or quiescent conditions, and at the time of ovulation or early transformation. According to this model, stromal cell-produced TGF-β normally maintains OSE cell growth in a quiescent state by its direct inhibitory actions on cell proliferation and

Figure 23: Model for OSE-thecal/stromal cell interactions under normal/quiescent conditions and at the time of ovulation, or of early transformation


Quiescent
normal OSE
LH-R
KIT
Basement
membrane
KIT/KL
proliferation
Epithelial cell
Stromal/thecal cell
Ovulation (LH surge) and
early transformation
(abberant TGF-β
signaling)
LH surge
KIT/KL
proliferation
LH surge
Epithelial cell
Stromal/thecal cell

indirectly by keeping Kit and KL expression in OSE low. At the time of ovulation, the LH surge downregulates TGF-β production by stromal/thecal cells, thereby relieving its inhibitory effect on OSE cell growth and on Kit and KL expression. Both the decrease in stromal cell TGF-β production and increase in Kit and KL signalling in OSE at this time lead to increased OSE cell proliferation for wound repair. The increase in KL expression also stimulates stromal/thecal cell proliferation, further repairing the ovulatory wound, or perhaps contributing to formation of the corpus luteum. Defective TGF-β signalling as might occur during early transformation of OSE cells will similarly lead to increased OSE cell proliferation, both directly and via increased Kit expression and signalling, resulting in the formation of invaginations and inclusion cysts. The LH surge can also act directly on the OSE to both increase both proliferation and KL expression, further contributing to wound repairing of OSE. A prolonged exposure to LH as seen in the menopausal ovary, might therefore contribute to the formation of invaginations and inclusion cysts by persistent stimulation of OSE cell proliferation. The role played by TGF-α/EGF in the proposed model remains unknown at this time.

The data presented in this project demonstrate that exogenous stromal/thecal-derived factors, SCM and gonadotropins can stimulate or inhibit the growth of OSE cells. The entrapment of OSE cells within the ovarian stroma as occurs following formation of inclusion cysts, could therefore expose OSE cells to these or other factors or concentrations which are not usually present at the ovarian surface thereby deregulating their growth. Transformation of OSE cells may occur as a result of accumulated mutations due increased cell division as in repeated ovulation, or due to the exposure to factors in the ovarian stroma not normally present at the ovarian surface and may allow

the OSE cells to escape the growth inhibitory effects of stromal/thecal-secreted substances thereby contributing to their uncontrolled growth leading to tumorigenesis. A bi-cameral culture system of OSE-stromal/thecal cells might better reflect the in vivo situation and better address the issues of stromal/thecal-secreted substances and their role in regulating OSE growth and induction of Kit expression in OSE cells.

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