THE ROLE OF TRANSFORMING GROWTH FACTOR BETA SIGNALING IN HUMAN OVARIAN CANCER CELLS

by

Lesley D. Dunfield

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

at

Dalhousie University
Halifax, NS
June, 2003

Your file Votre référence
ISBN: 0-612-83712-2
Our file Notre référence
ISBN: 0-612-83712-2

DALHOUSIE UNIVERSITY

DEPARTMENT OF PHARMACOLOGY

The undersigned hereby certify that they have read and recommend to the Faculty of Graduate Studies for acceptance a thesis entitled "The role of transforming growth factor beta signaling in human ovarian cancer cells" by Lesley Dunfield in partial fulfillment for the degree of Doctor of Philosophy.

Dated: June 9, 2003

External Examiner:

Research Supervisor:

Examining Committee:

Departmental Representative:

DALHOUSIE UNIVERSITY

Date: June 9, 2003

Author: Lesley D. Dunfield

Title: The role of transforming growth factor beta signaling in human ovarian cancer cells

Department: Pharmacology

Degree: PhD Convocation: Fall Year: 2003

Permission is herewith granted to Dalhousie University to circulate and to have copied for non-commercial purposes, at its discretion, the above title upon the request of individuals or institutions.

Signature of author

The author reserves other publication rights, and neither the thesis nor extensive extracts from it may be printed or otherwise reproduced without the author's written permission.

The author attests that permission has been obtained for the use of any copyrighted material appearing in the thesis (other than the brief excerpts requiring only proper acknowledgement in scholarly writing), and that all such use is clearly acknowledged.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Ovarian cancer is the most lethal of the gynecologic cancers. The majority of ovarian cancers arise from the ovarian surface epithelium (OSE). Transforming growth factor beta (TGFß) inhibits proliferation of the OSE, which secretes and expresses receptors for TGFß. Resistance to the anti-proliferative effect of TGFß is demonstrated in numerous cancers, due to defects in the TGFß signaling pathway. TGFß signals through a receptor complex, which activates intracellular signaling molecules known as Smads. Activated Smads translocate to the nucleus, interact with other transcription factors, and modulate target gene expression.

This study investigated the TGFß signaling pathway in primary human ovarian cancer cells. All components of the TGFß signaling pathway are expressed and functional in primary ovarian cancer cells. Furthermore, primary ovarian cancer cells respond to the anti-proliferative effect of TGFß, which is partly mediated by TGFß-induced up-regulation of $p15^{INK4B}$. Although primary ovarian cancer cells respond to the anti-proliferative effect of TGFß in cell culture, ovarian cancer cells *in vivo* are exposed to TGFß but continue to proliferate. Therefore, mechanisms must exist to inhibit the anti-proliferative effect of TGFß contributing to uncontrolled cellular proliferation *in vivo*.

Numerous signaling pathways can converge with the TGFß signaling pathway to modulate its effects. Epidermal growth factor (EGF) is mitogenic to ovarian cancer cells, and can modulate TGFß signaling. This study demonstrates that EGF inhibits the anti-proliferative effect of TGFß in primary ovarian cancer cells. TGFß-induced up-regulation of $p15^{INK4B}$ is decreased by EGF, which likely accounts for the EGF inhibition of the anti-proliferative effect of TGFß. Therefore, although the TGFß signaling pathway remains functional in primary ovarian cancer cells, inhibition of the anti-proliferative effect of TGFß by factors such as EGF may occur *in vivo*. Inhibition of these factors may reverse resistance to TGFß and may be a potential therapy for ovarian cancer patients.

ABBREVIATIONS

BAMBI – BMP and activin membrane bound inhibitor

ß-gal – Beta-galactosidase

BMP – Bone morphogenetic protein

cDNA – complimentary DNA

CLL – Chronic lymphocytic leukemia

Co-Smad – Common mediator Smad

DHT – 5α-dihydrotestosterone

DMEM – Dulbecco's modified eagles medium

DMSO – Dimethylsulfoxide

DNA – Deoxyribonucleic acid

dpp – Decapentaplegic

ECL – Enhanced chemiluminescence

ECM – Extracellular matrix

EGF – Epidermal growth factor

ELISA – Enzyme linked immunosorbent assay

EMT – Epithelial to mesenchymal transition

ER – Estrogen receptor

FBS – Fetal bovine serum

FGF – Fibroblast growth factor

FIGO – International federation of gynecologic oncologists

GAPDH – Glyceraldehyde-3-phosphate dehydrogenase

HDAC – Histone deacetylase

HGF – Hepatocyte growth factor

HHT – Hereditary hemorrhagic telangiectasia

HRP – Horse radish peroxidase

ID1 – Inhibitor of differentiation

IFNγ – Interferon gamma

IGF-1 – Insulin like growth factor 1

IL - Interleukin

I-Smad – Inhibitory Smad

LAP – Latency associated peptide

LTBP – Latent TGFß binding protein

MAD – mothers against Dpp

MAPK – Mitogen activated kinase

MH1 – Mad homology 1

MH2 – Mad homology 2

MIS – Mullerian inhibiting substance

MISIIR – MIS type II receptor

mRNA – messenger RNA

MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyltetrazolium bromide

NES – Nuclear export signal

NLS – Nuclear localization signal

OC – Ovarian cancer

ONPG – o-nitrophenyl ß-D-galactopyranoside

OSE – Ovarian surface epithelium

PAI-1 – Plasminogen activator inhibitor

PBS – Phosphate buffered saline

PDGF – Platelet derived growth factor

POD – Peroxidase

PR – Progesterone receptor

RNA – ribonucleic acid

R-Smad – Receptor-activated Smad

RT-PCR – Reverse transcriptase polymerase chain reaction

SEM – Standard error of the mean

SARA – Smad anchor for receptor activation

SDS-PAGE – Sodium dodecylsulfate-polyacrylamide gel electrophoresis

SSC – Standard saline citrate

SV40-TAg – Simian virus 40 T antigen

TßRI – TGFß receptor type I

TßRII – TGFß receptor type II

TBS – Tris buffered saline

TBS-T – Tris buffered saline + tween

TCA – Trichloroacetic acid

TF – Transcription factor

TGFα - Transforming growth factor alpha

TGFß – Transforming growth factor beta

TNFα - Tumor necrosis factor alpha

TPA - 12-*O*-tetradecanoyl-13-acetate

tPA – Tissue type plasminogen activator

TRAP – TßRI associated protein

TRE – TPA response element

TRIP – TGFß receptor interacting protein

TSA – Trichostatin A

TSP-1 – Thrombospondin 1

uPA – Urokinase plasminogen activator

VEGF – Vascular endothelial growth factor

ACKNOWLEDGEMENTS

Things I learned in University: how to make an elephant out of a clementine peeling, "laughter is the key to acceptance", wine appreciation, who will help me t.p. the boss's office, glass painting, which mop works the best, paper WILL catch on fire in the microwave, and most importantly: why i-mac's have handles. Oh – and I learned a bit about science too.

First of all, I wish to thank my supervisor, Mark Nachtigal. When I approached you as a 4th year undergraduate student with very little lab experience - you didn't hesitate to take me on as a grad student – thank you for taking a chance on me, and for all the advice, support, and guidance. I also wish to acknowledge the Rossetti Studentship and Cancer Research and Education-Nova Scotia for funding throughout my studies.

Diane- you've been a great lab mate and friend and I'm so glad we've been able to work together. Thanks for everything – it won't be the same doing bench work without you across from me. And I couldn't have asked for better post-docs to work with - Yangxin and Trevor, you are both amazing, and I can't imagine how I would have managed without the two of you. You've been incredibly helpful and I've had so much fun working with you both. Thanks also to Gaby for all your help and input, and to Elizabeth who showed me the ropes when I first started. A special thanks to Luisa, Janet, and Sandi. You never came through with the threat to kick me out of the office when I was harassing you days on end with various questions and 'issues'. Thanks for your patience and kindness.

Grad school would not have been the same without Michelle! Having you down the hall (although you seemed to be in 5B an awful lot!) has been great, and you have no idea what a huge part you have played in this whole experience. You have taught me so much (scientific and otherwise – I won't elaborate for fear of incriminating myself.☺). Thank you for everything, and I honestly don't know what I'm going to do without you.

A huge thank you to my parents - you've been incredibly supportive throughout this whole fiasco and now the "little paper I've been working on" is done. Thanks also to the rest of my family as well – my sister and family and my Grandparents. Sorry for all the missed family functions, and thanks for understanding! Now the "when will you be done school" questions can finally end!

CHAPTER 1

GENERAL INTRODUCTION

1.1 Ovarian Cancer

There will be approximately 2600 new cases of ovarian cancer diagnosed in Canada in 2003, and about 1550 deaths from this disease (Canadian Cancer Society 2003 Statistics). Ovarian cancer is the leading cause of death among the gynecologic cancers (Tortolero-Luna et al. 1994). There are no effective early detection methods for ovarian cancer, therefore, it is typically not diagnosed until late stages, after metastasis has occurred. The majority of ovarian cancers (~90 %) are epithelial carcinomas, arising from the ovarian surface epithelial cells (OSE) (Tortolero-Luna et al. 1994). Ovarian stromal tumors and germ cell tumors account for the remaining incidences of ovarian cancer.

1.1.1 OSE biology

The majority of ovarian cancers arise from the OSE. The OSE is the layer of squamous to cuboidal epithelium that covers the surface of the ovary and is a modified mesothelium, which has mesenchymal and epithelial characteristics (Auersperg et al. 1994; Auersperg et al. 2001). Early in development, the OSE is part of the celomic epithelium which differentiates to form oviductal, endometrial and endocervical epithelium, as well as OSE (Auersperg et al. 2001). It appears, however, that the OSE is less differentiated than these other types of epithelium, due to lack of expression of the differentiation marker CA-125 (Auersperg et al. 2001). Epithelial ovarian cancers express CA-125, which therefore indicates that the OSE has the ability to differentiate (Auersperg et al. 2001).

A basement membrane separates the OSE from the underlying stroma. The OSE is weakly adhered to the basement membrane, and is easily removed and therefore often absent in ovarian samples. The epithelial markers keratins 7, 8, 18, 19 as well as the

mesenchymal marker vimentin are expressed by the OSE (Auersperg et al. 2001). OSE can also undergo epithelial-mesenchymal transition (EMT), which likely plays a role in post-ovulatory repair (Auersperg et al. 2001). Functions of the OSE include transport of materials to and from the peritoneal cavity and repair of the ovarian surface after ovulation (Auersperg et al. 1994; Auersperg et al. 2001).

1.1.2 Pathology of epithelial ovarian cancer

During ovarian cancer development, the OSE differentiates to resemble the epithelium of other gynecologic tissues. The majority (80%) of epithelial ovarian cancers are serous adenocarcinomas. These tumors arise by differentiation of the OSE to resemble fallopian tube epithelium. Mucinous tumors resemble the epithelium of the endocervix, endometrioid tumors resemble endometrial epithelium, clear-cell tumors resemble epithelium of the endometrium during pregnancy, and undifferentiated tumors do not have characteristics of a particular gynecologic tissue (Scully 1995; Auersperg et al. 2001). The International Federation of Gynecologic Oncologists (FIGO; 1971) classifies epithelial ovarian cancer into 4 stages. Stage I tumors are confined to the ovaries; IA involves one ovary, IB involves both ovaries, and IC also presents with cancer cells in peritoneal fluid or malignant ascites. Ovarian tumors with pelvic extension are stage II tumors, with extension to the uterus, fallopian tubes or other pelvic organs and the presence or absence of cancer cells within ascites (IIA-IIC). Stage III ovarian cancers include tumors outside of the pelvis or positive nodes, and Stage IV cancers involve distant organs, such as the liver parenchyma or pleural space (FIGO 1971).

1.1.3 Ovarian cancer risk factors

Ovarian cancer risk factors include nulliparity, age, and family history. In 1971, Dr. M.F. Fathalla proposed the "incessant ovulation" hypothesis to explain the occurrence of ovarian tumorigenesis (Fathalla 1971). Fathalla hypothesized that disruption and repair of the ovarian epithelium after repeated ovulations presents a greater risk for malignant transformation. In accordance with this hypothesis, absence of ovulation should decrease risk of ovarian cancer. Indeed, risk factors such as nulliparity and age can be correlated with increased number of ovulations. The majority of epithelial ovarian cancers occur in women aged 60 – 70 (Tortolero-Luna et al. 1994), whereas germ cell tumors usually occur in woman aged 15 – 24, and stromal tumors occur with an age pattern similar to that of the epithelial cancers (Tortolero-Luna et al. 1994). Although family history is a risk factor, only about 5% of ovarian cancers are familial (Tortolero-Luna et al. 1994).

Multiparity, breast feeding, and oral contraceptive use are all associated with a decreased risk for ovarian cancer. Parity has been found to decrease risk of ovarian cancer (Risch et al. 1983; Whittemore et al. 1992; Hanikinson et al. 1995). Women with one pregnancy had a decrease in risk, and multiparity decreased risk by 45% compared to nulliparity (Hankinson et al. 1995). A first pregnancy decreases risk to a greater extent than additional pregnancies, and each additional pregnancy decreases risk by 14% (Tortolero-Luna et al. 1994). Similarly, breast feeding has also been identified as a protective factor in ovarian cancer (Risch et al. 1983; Whittemore et al. 1992). Woman who breast feed have a 40% decreased risk compared to women with children who do not breast feed, and a 60% decreased risk compared to woman without children (Tortolero-

Luna et al. 1994). Hence, the combination of pregnancy and breast feeding provides significant protection against ovarian cancer.

Numerous studies have investigated the use of oral contraceptives and incidence of ovarian cancer, and have all found that risk decreases with use (Weiss et al. 1981; Rosenberg et al. 1982; Whittemore et al. 1992; Rosenberg et al. 1994). Some studies have found that extended use decreases risk (Royar et al. 2001; Bosetti et al. 2002), whereas others have shown that as little as 3 months use decreases risk, and the benefit continues for 10 – 15 years following discontinuation of use (Rosenberg et al. 1982; Royar et al. 2001). It has been suggested that low dose oral contraceptive use has a greater effect on risk reduction compared to high dose (Royar et al. 2001), but an explanation for this finding was not suggested. In contrast, however, Schildkraut et al. (2002) found oral contraceptives containing high levels of progestin were more effective at decreasing risk for ovarian cancer compared to those with low dose progestin; estrogen dose had no effect (Schildkraut et al. 2002).

Although the incessant ovulation hypothesis offers an explanation as to why oral contraceptive use is protective against ovarian cancer considering oral contraceptives inhibit ovulation, this hypothesis has been challenged. Anovulation may not be the cause of the protective effect of oral contraceptives. Studies suggest that oral contraceptives induce apoptosis of OSE cells and rid the body of potentially tumorigenic cells (Rodriquez et al. 1998). Indeed, apoptosis has been found to be induced by progestin *in vivo* using a Macaque monkey model (Rodriquez et al. 2002). The induction of apoptosis in the OSE appears to be mediated by a change in expression patterns of transforming growth factor beta (TGFß) isoforms, in that, increased expression of TGFß2/3 and

decreased expression of TGFß1 in response to the progestin component of oral contraceptives results in the induction of apoptosis (Rodriquez et al. 2002), which may contribute to the protective effects of oral contraceptives. Estrogen was not found to affect OSE apoptosis.

1.1.4 Screening for epithelial ovarian cancer

There are currently no effective early detection methods for ovarian cancer, and for this reason, greater than 70% of ovarian cancers are diagnosed after metastasis has occurred (Tortolero-Luna et al. 1994). Bast et al. (1981) initially described an antibody, OC125, which reacts with a tumor associated antigen on epithelial ovarian cancer cell lines and ovarian tumor tissue, and did not react with other cancer types (Bast et al. 1981). The tumor associated antigen recognized by OC-125 was later described as CA-125, which is a high molecular weight serum glycoprotein complex (Davis et al. 1986; Bast et al. 1983). Although measuring CA-125 levels can be helpful in monitoring disease progression and response to treatment, its usefulness as a diagnostic method is limited. Some ovarian tumor types do not express CA-125. For example, CA-125 was detected in 19/23 serous, 2/3 endometrioid, 1/4 clear cell, and 2/2 undifferentiated ovarian carcinomas, however, CA-125 was not detected in 8 mucinous ovarian carcinomas (Kabawat et al. 1983). In addition, although levels of CA-125 are increased in advanced stages of ovarian cancer patients, only 50-60% of stage I patients have elevated CA-125 levels (Hempling 1994). CA-125 has also been detected in bowel, breast, lung, and pancreatic cancer, as well as in pregnancy, pelvic inflammatory disease, endometriosis, cirrhosis, and pancreatitis (Tortolero-Luna et al. 1994). Combining measurement of CA-125 levels with sonography can enhance specificity (Tortolero-Luna

et al. 1994), but clearly, improved diagnostic methods for early detection of ovarian cancer are necessary.

Recently, Petricoin et al. (2002) have investigated the use of proteomics to detect ovarian cancer. A proteomic pattern was identified, and analysis of serum from ovarian cancer patients and control samples correctly identified all ovarian cancer patients, including 6 patients with stage I ovarian cancer. Furthermore, 63/66 of the control samples were correctly detected as non-cancerous (Petricoin et al. 2002). Control samples included women with no ovarian cysts, benign ovarian cysts, benign gynecological disease, and non-gynecological inflammatory disorder. The 3 false positives were samples with no ovarian cysts, and samples with benign cysts < 2.5cm. Clearly, the development of a test to identify a proteomic pattern will be an important development for early detection of ovarian cancer and will likely improve patient outcome.

1.1.5 Treatment and prognosis

Surgery and chemotherapy are used in the treatment of ovarian cancer. Patients with early stages of ovarian cancer undergo salpingo-oophorectomy, which may be bilateral, or unilateral if the woman is of child-bearing age (Gibbs & Gore 2001). Hysterectomy, oophorectomy, and omentectomy are performed on ovarian cancer patients at late stages of the disease. Whether chemotherapy is also administered depends on the stage of the cancer. Low stage and low grade ovarian cancer patients may not require chemotherapy, but patients with higher stages typically are administered chemotherapy. Decreasing tumor burden by surgery is also performed on later stage ovarian cancer patients (Gibbs & Gore 2001).

Traditionally, platinum-containing chemotherapeutic agents are the first-line treatment for ovarian cancer patients. Recently, however, taxanes have been approved for the treatment of ovarian cancer, and paclitaxel in combination with platinum is now the common therapy for ovarian cancer patients (Gibbs & Gore 2001; Ozols 2002). In addition, cyclophosphamide, topotecan, encapsulated doxorubicin, and gemcitabine have also been tested for use in ovarian cancer patients, and may also be used in combination with platinum agents (du Bois 2001; Ozols 2002;).

Unfortunately, the prognosis for ovarian cancers patients is seldom favorable. Lack of early detection methods and effective treatments for advanced stages of the disease contribute to the poor prognosis. The five-year survival rate for ovarian cancer is only about 20%, however, if the ovarian cancer is detected at an early stage, prognosis is much better. These patients can have a 5-year survival rate of 80% - 90%. The development of effective early detection methods as well as more useful therapeutics are critical to enhance patient survival.

1.1.6 Epithelial ovarian tumorigenesis

The "incessant ovulation" hypothesis was widely accepted as the cause of ovarian tumorigenesis (Falthalla 1971), however, this hypothesis has been challenged based on studies investigating the occurrence of inclusion cysts. The OSE can form inclusion cysts in the ovarian stroma, and it has been suggested that these inclusion cysts can be the sites of ovarian tumor development (Blaustein et al. 1982; Scully 1995). Therefore, if the incessant ovulation hypothesis were correct, a greater number of cysts would be expected in nulliparous women. Actually, a greater number of cysts have been found in multiparous women, as well as in women who have decreased or no ovulation (Scully

1995). Therefore, the formation of inclusion cysts is not dependent on ovulation, which suggests that incessant ovulation hypothesis may not be solely responsible for ovarian tumorigenesis.

Inclusion cysts likely form as a result of proliferation of the OSE and stroma and pinching off of the OSE to form OSE lined cysts within the stroma (Scully 1995). Metaplasia can often be found in ovarian inclusion cysts contralateral to cancerous ovaries (Resta et al. 1993; Scully 1995), and this metaplasia is more common in the OSE of inclusion cysts, compared to the OSE on the ovarian surface (Resta et al. 1993). In addition, CA-125 is more commonly expressed in the OSE cells of inclusion cysts, compared to OSE on the surface of the ovary (Scully 1995). Hence, the OSE within inclusion cysts is likely exposed to factors that promote tumor formation. This may be due to the fact that OSE within inclusion cysts is not separated from the stroma by the basement membrane and therefore may be exposed to tumor promoting factors produced by the stroma acting in a paracrine manner. Furthermore, factors produced by the OSE may act in an autocrine fashion to promote tumorigenesis, which may be found at higher levels within the enclosed space of an inclusion cyst, as opposed to factors produced by the OSE on the surface of the ovary (Auesperg et al. 2001).

Although normal OSE exhibits both epithelial and mesenchymal characteristics, neoplastic OSE takes on the epithelial phenotype (Auesperg et al. 1998). Normal OSE do not express the epithelial cell adhesion molecule E-cadherin, however, ovarian cancer cells can express E-cadherin (Auersperg et al. 1994). In fact, overexpression of E-cadherin in normal OSE induces epithelial characteristics, such as growth in a cobblestone monolayer culture and expression of CA-125 (Auersperg et al. 1999).

Hence, E-cadherin may play a role in transformation of OSE. Furthermore, ovarian cancer cell lines have been developed by transfection of E-cadherin and simian virus 40 T antigen (SV40 TAg) into normal OSE (Ong et al. 2000), and these cells exhibit characteristics of other ovarian cancer cell lines, further implying that E-cadherin expression may play a role in OSE transformation.

Metastasis of ovarian cancer occurs by exfoliation of tumor cells into the peritoneal cavity, and transportation of the cancer cells through the peritoneal fluid. Abdominal surfaces are exposed to the cancer cells within the peritoneal fluid, and ovarian cancer cells attach resulting in the formation of secondary tumors on peritoneal surfaces (Hoskins 1993). The most common site of ovarian cancer metastasis is the omentum (Hoskins 1993). Ascites fluid, the excessive amount of peritoneal fluid containing cancer cells, can be a symptom of late stage ovarian cancer, as well as other gynecologic disease. Two opposing mechanisms have been proposed to explain ascites fluid accumulation. First, hyperpermeability of the vasculature of peritoneal surfaces has been described (Straube 1958; Senger et al. 1983), whereas lymphatic block caused by proteins and erythrocytes inhibiting drainage of peritoneal fluid is also a proposed mechanism of ascites accumulation (Straube 1958). Ascites fluid contains cancer cells, mononuclear cells, and macrophages (Stratton & DiSaia 1981), and is a useful source of ovarian tumor cells for experimentation.

Ovarian cancer research is conducted using either ovarian cancer cell lines or primary ovarian cancer cells isolated from either tumor samples of ovarian cancer patients, or isolated from ascites fluid. The usefulness of ovarian cancer cell lines is limited due to the genetic changes present in cell lines which may affect results,

particularly results from experiments examining proliferation. Data obtained from experiments using cell culture is restricted, however, the lack of a mouse model of ovarian cancer has made investigations using cell culture techniques necessary. Recently, however, Connolly et al. (2003) describe a transgenic ovarian cancer mouse model. SV40 TAg expressed under control of the mullerian inhibiting substance (MIS) type II receptor (MISIIR) promoter was utilized to specifically express oncogenic SV40 TAg in the ovary of mice. Epithelial ovarian tumors were developed in 50% of the transgenic MISIIR-TAg mice and ascites fluid was typically present as well (Connolly et al. 2003). Clearly, development of an ovarian cancer mouse model will greatly benefit the field of ovarian cancer research and lead to a better understanding of the processes that contribute to ovarian tumorigenesis.

1.1.7 Regulation of ovarian cancer cell proliferation

Hormones, growth factors and cytokines play a role in OSE regulation. The OSE secretes ligands and expresses receptors for various factors which function to modulate proliferation of the OSE. Normal OSE and ovarian cancer cells express receptors for lutenizing hormone (LH) and follicle stimulating hormone (FSH), as well as gonadotropin releasing hormone (GnRH) (Zheng et al. 1996; Mandai et al. 1997; Kang et al. 2000; Zheng et al. 2000; Auesperg et al. 2001). GnRH is produced by the OSE and acts in an autocrine manner to inhibit proliferation of normal OSE as well as ovarian cancer cells (Emons et al. 1993; Kang et al. 2000). LH and FSH have been shown to stimulate proliferation of rabbit OSE (Osterholzer et al. 1985), and FSH was shown to stimulate proliferation of primary human ovarian cancer cells, which was inhibited by co-treatment with LH (Zheng et al. 2000).

Although the estrogen and progesterone receptors are expressed in normal OSE (Karlan et al. 1995), receptor expression in ovarian cancer cells is unclear. Estrogen receptor (ER) and progesterone receptor (PR) expression has been described in ovarian cancer cells, however, inconsistencies are reported. Anderl et al. show 63% of ovarian tumors express ER and 38% express PR, whereas lower expression is seen in benign tumors (Anderl et al. 1988). A greater number of serous ovarian cancers were found to express ER compared to other histologies, and overall, 33% of ovarian cancers expressed both ER and PR, whereas 40% expressed neither ER nor PR (Harding et al. 1990). Ford et al. found 50% of well-differentiated serous ovarian cancers expressed ER, whereas no poorly differentiated cancers expressed ER or PR (Ford et al. 1983). Similarly, 86% of well differentiated ovarian tumors expressed ER and only 23% of poorly differentiated ovarian tumors expressed ER (Iverson et al. 1986). Overall, the role that estrogen and progesterone play in ovarian tumorigenesis is unclear, although it is well established that oral contraceptive use is protective against ovarian cancer.

Cytokines, such as interleukin-1 (IL-1), IL-6 and tumor necrosis factor alpha (TNFα) are produced by normal and tumorigenic OSE (Auesperg et al. 2001). Peritoneal fluid of ovarian cancer patients contains IL-1, IL-6, interferon gamma (IFNγ) and transforming growth factor alpha (TGFα), and IL-6 levels were higher in ovarian cancer samples compared to other gynecologic cancers (Punnonen et al. 1998). Epithelial ovarian cancer samples were shown to express IL-1, IL-6, TNFα among other cytokines, however, IFNγ was not expressed (Burke et al. 1996). It has been suggested that IL-1 and IL-6 stimulate proliferation and induce metastasis of ovarian cancer cells, and therefore may play a role in ovarian cancer progression (Malik & Balkwill 1991;

Auesperg et al. 2001). Although TNFα stimulates proliferation of normal OSE, it has been shown to inhibit proliferation of ovarian cancer cells (Marth et al. 1996). Due to the cytokine expression profile in peritoneal fluid of ovarian cancer patients and the proliferative responses, cytokines likely play a role in ovarian cancer progression.

Hepatocyte growth factor (HGF) may play a role in ovarian tumorigenesis, as it has been found to be present in ascites fluid of ovarian cancer patients (Sowter et al. 1999). In addition, the HGF receptor, Met, has been shown to be overexpressed in ovarian cancer (DiRenzo et al. 1994). HGF is mitogenic to both normal OSE and ovarian cancer, and induces migration of ovarian cancer cell lines (Corps et al. 1997; Ueoka et al. 2000). Other growth factors such as basic fibroblast growth factor (bFGF; Crickard et al. 1994), platelet derived growth factor (PDGF; Henrikson et al. 1993; Dabrow et al. 1998), insulin like growth factor 1 (IGF-1; Yee et al. 1991; Beck et al. 1994; Conover et al. 1998), and vascular endothelial growth factor (VEGF; Abu-Jawdeh et al. 1996) have been shown to be expressed and modulate proliferation of ovarian cancer cells.

Epidermal growth factor (EGF) also stimulates proliferation of normal OSE and ovarian cancer cells (Siemens & Auesperg, 1988; Rodriguez et al. 1991; Crew et al. 1992; Berchuck et al. 1993). EGF was found to be expressed in 25% - 49% of ovarian cancer samples (Burke et al. 1996), and in 27.6% in an independent study (Owens et al. 1991a). TGFα is an EGF homolog which acts through the EGF receptor. Numerous studies have found TGFα expression in ovarian cancer samples (Morishige et al. 1991; Owens et al. 1991a; Kohler et al. 1992; Burke et al. 1996; Chien et al. 1997; Saltzman et al. 1999), and studies have shown TGFα stimulates proliferation of human ovarian cancer cells (Morishige et al. 1991; Crew et al. 1992; Stromberg et al. 1992). TGFα was

expressed in 88.5% and 90% of ovarian cancer samples in independent studies (Owens et al. 1991a; Burke et al. 1996). The EGF receptor (EGFR) is also expressed in 35% - 75% of ovarian cancers (Battaglia et al. 1989; Berchuck et al. 1991; Morishige et al. 1991; Owens et al 1991b; Kohler et al. 1992) and expression can be higher in ovarian tumors compared to normal OSE and benign tumors (Berns et al. 1992). In addition, the EGFR homolog, HER2/neu, has been shown to be expressed in over 30% of ovarian cancers but the association of HER2/neu with poor prognosis is unclear (Slamon et al. 1989; Haldane et al. 1990). Although Haldane et al. (1990) suggests HER2/neu expression does not affect patient prognosis, Slamon et al. (1989) suggest that overexpression of HER2/neu is associated with poor patient prognosis. Overall, based on receptor expression studies and the proliferative effect, EGF and TGFα signaling may play an important role in ovarian tumorigenesis.

The OSE has been demonstrated to be growth arrested by TGFß (Berchuck et al. 1992). TGFß is produced by the OSE, which also bears receptors for the ligand (Henriksen et al. 1995). Ovarian cancer cell lines are resistant to the anti-proliferative effect of TGFß (Berchuck et al. 1992), whereas reports have shown both sensitivity and resistance in primary ovarian cancer cells (Hurteau et al. 1994; Yamada et al. 1999). Loss of responsiveness to the anti-proliferative effect of TGFß occurs in other types of cancers, and therefore, may contribute to ovarian cancer progression.

1.2 TGFß

TGFß is a member of a superfamily of secreted growth factors, which includes activin, bone morphogenetic proteins (BMP), growth and differentiation factors, as well as the

distant members MIS and inhibin (Massagué 1998). Monomeric TGFß is 112 amino acids, which encodes a 12.5 kDa protein (Derynck et al. 1985). TGFß was first identified due to its transforming activity in rat kidney fibroblast cells (Roberts et al. 1981). Endothelial cells, epithelial cells and cells of the immune system secrete and are acted on by TGFß, which has a number of different functions, such as cellular proliferation, differentiation, immunosuppression, angiogenesis, extracellular matrix (ECM) formation and wound healing (Roberts et al. 1981; Kerhl et al. 1986; Masui et al. 1986; Roberts et al. 1986; Yu & Stamenkovic 2000).

1.2.1 TGFß isoforms

There are 3 mammalian TGFß isoforms; TGFß1-3. These 3 isoforms share approximately 80% amino acid identity, they are encoded by different genes located on different chromosomes, and although their activities are similar, their expression patterns vary (Gold 1999; Piek et al. 1999). TGFß isoform expression varies in development, regeneration and pathological responses (Roberts & Sporn 1992). TGFß3 is 50 times more potent at inhibiting proliferation of epithelial cells compared to TGFß1 or TGFß2, whereas TGFß1 and TGFß3 are both 100 times more potent than TGFß2 at inhibiting proliferation of vascular endothelial cells (Graycar et al. 1989; Merwin et al. 1991). Mice deficient in TGFß1 typically are embryonic lethal due to vascular and hematopoietic abnormalities, however, some survive but die early due to lymphocyte and macrophage infiltration into organs (Shull et al. 1992; Kulkarni et al. 1993; Diebold et al. 1995). Multiple developmental abnormalities occur in TGFß2 deficient mice, leading to perinatal death, whereas deficient pulmonary development and palate development occur

in TGFß3 mice, also causing early death (Kaartinen et al. 1995; Proetzel et al. 1995; Sanford et al. 1997).

1.2.2 Activation of latent TGFß

TGFß is secreted as an inactive precursor. Active TGFß is in the form of a 25 kDa homodimer, with the 12.5 kDa TGFß monomers linked by a disulfide bond. Latent TGFß consists of the 25 kDa TGFß dimer non-covalently associated with latency associated peptide (LAP) (Assoian et al. 1983; Miyazono et al. 1988; Wakefield et al. 1988; Wakefield et al. 1990; Miyazono et al. 1991). In addition, latent TGFß binding protein (LTBP) can also be associated with this complex (Figure 1-1). LTBP is linked to LAP by a disulfide bond, and plays a role in secretion, storage within the ECM, and activation of TGFß (Assoian et al. 1983; Wakefield et al. 1988; Miyazono et al. 1988; Miyazono et al. 1991; Nunes et al. 1997; Taipale et al. 1994). LTBP is not required for retaining TGFß in a latent form; rather it serves to bind TGFß to ECM for proteolysis (Taipale et al. 1994; Nunes et al. 1997). Latency of TGFß is achieved by association with LAP, which inhibits TGFß receptor binding.

In vitro activation of TGFß can be achieved by extreme heat or pH; however, *in vivo* activation mechanisms are not thoroughly understood. Dubois et al. (1995) conducted *in vitro* assays and showed that furin cleaves pro-TGFß1 to efficiently produce active TGFß1. Furthermore, colon carcinoma cells deficient in furin are unable to process TGFß, whereas introduction of furin into these cells results in increased amounts of active TGFß1 (Dubois et al. 1995). The adhesion protein thrombospondin 1 (TSP-1) associates with and can activate TGFß *in vitro* and in cell culture (Schultz-Cherry & Murphy-Ullrich 1993). Experiments stemming from the observation that mice lacking

TGFß1 displayed a similar phenotype compared to TSP-1 null mice resulted in pharmacological investigations to show that TSP-1 can activate TGFß1 *in vivo* (Crawford et al. 1998). Additional activation mechanisms must be present *in vivo* however, since TSP-1 null mice do not die as early as TGFß1 null mice (Crawford et al. 1998).

Proteases other than furin have also been shown to activate TGFß. Calpain, a cysteine protease, can activate latent TGFß *in vitro* and in cell culture (Abe et al. 1998). Plasmin and cathepsin are proteases that have been described to activate latent TGFß *in vitro*. Plasmin may activate TGFß by releasing the latent TGFß from the ECM, and by proteolytically cleaving the latent TGFß to form active TGFß (Lyons et al. 1990). Cathepsin cleaves latent TGFß1, and the resulting active TGFß1 inhibits cell growth in mink lung epithelial cells, indicating that cathepsin can activate latent TGFß1 (Somanna et al. 2002). Although numerous mechanisms have been suggested to activate TGFß physiologically, an obvious single mechanism is not yet defined, and TGFß is likely activated physiologically by a combination of factors.

1.2.3 TGFß receptors

Once latent TGFß has been activated, it is able to bind the TGFß receptors. The TGFß receptors are transmembrane serine/threonine kinase receptors, and include TGFß type I (TßRI) and TGFß type II (TßRII) receptors (Figure 1-2). TßRI is approximately 55 kDa, whereas TßRII is approximately 70 kDa (Massagué 1998). TGFß initially binds to TßRII, which is a constitutively active kinase. TGFß1 and TGFß3 bind TßRII at a higher affinity than TGFß2 (Massagué 1992). TßRII is phosphorylated at multiple serine residues by cellular kinases as well as by autophosphorylation, in the presence and absence of ligand (Wrana et al. 1994). Once ligand binds to TßRII, TßRI is recruited,

and is phosphorylated by TßRII. TßRII phosphorylates several residues in the GS domain of TßRI (Figure 1-2). This activates TßRI kinase activity to initiate the TGFß signaling events downstream, which are mediated by intracellular signaling molecules known as Smads (Wrana et al. 1994; Souchelnytskyi et al. 1996; Heldin et al. 1997).

Betaglycan is also known as the TGFß type III receptor (TßRIII) and binds all 3 isoforms of TGFß, acting as an accessory to aid in ligand binding to TßRII. TGFß is presented to TßRII by betaglycan which acts to regulate ligand binding and signaling (Lopez-Casillas et al. 1993). Interestingly, TGFß2 has low affinity for TßRII, and actually requires betaglycan for TßRII binding (Rodriguez et al. 1995). Endoglin is another accessory receptor similar to betaglycan, which binds TGFß1 and TGFß3, but not TGFß2 (Lastres et al. 1996; Cheifetz et al. 1992). Endoglin is highly expressed on endothelial cells, and requires association of type I and type II receptors to bind ligand (Gougos & Letarte 1990; Cheifetz et al. 1992; Yamashita et al. 1994). The role of endoglin remains unclear, however, endoglin has been shown to modulate TGFß signaling, and is associated with altering angiogenesis and mutations in endoglin have been linked to hereditary hemorrhagic telangiectasia type I (HHT1) (McAllister et al. 1994; Lastres et al. 1996; Barbara et al. 1999).

1.2.4 TGFß receptor down-regulation

TGFß receptors are down-regulated by clathrin-mediated endocytosis. Homomeric receptors are recycled back to the membrane, whereas heteromeric receptors are degraded by lysosomes/proteosomes (Anders et al. 1997; Doré et al. 2001). TGFß receptors not bound to ligand are constantly being recycled, but upon ligand binding, the receptors are degraded (Doré et al 2001). Penheiter et al. (2002) found that endocytosis

of TGFß receptors was required for signaling. TßRI activation and association with Smad2 occurs in the absence of endocytosis, however, Smad activation is dependent on endocytosis (Penheiter et al. 2002). Therefore, receptor endocytosis serves 2 functions; to degrade receptors to reduce signaling activity, and to transmit the signal (Penheiter et al. 2002). Recently, however, Lu et al. (2002) explained that Smad2 activation can occur independently of receptor endocytosis (Lu et al. 2002). TGFß induced Smad2 activation, nuclear translocation and transcription are not blocked by inhibition of receptor endocytosis, however, it remains possible that the Smad2 response is diminished by blockage of receptor endocytosis (Lu et al. 2002). Recently, it has been shown that receptor endocytosis by both clathrin-mediated endocytosis and lipid rafts is necessary for TGFß signaling control (Di Guglielmo et al. 2003). Activation of TGFß signaling is mediated by internalization of TGFß receptors in clathrin vesicles, which are associated with SARA. Internalization of TGFß receptors into lipid rafts is associated with receptor degradation, which is mediated by Smurf and Smad7. Therefore, levels of clathrin and lipid rafts likely play a role in regulation of TGFß signaling and receptor down-regulation (Di Guglielmo et al. 2003).

1.2.5 TGFß receptor binding proteins

TGFß receptor activity can be altered by association with other proteins. Proteins have been identified using yeast two-hybrid screens which can bind to TGFß receptors to modulate activity. TGFß receptor interacting protein-1 (TRIP-1) associates with TßRII, whereas FKBP-12, serine-threonine kinase receptor-associated protein (STRAP), farnesyltransferase α, BMP and activin membrane-bound inhibitor (BAMBI), and TßRI associated protein-1 (TRAP-1) associate with TßRI to modulate TGFß signaling. TRIP-1

associates with TßRII, as well as the TßRII-TßRI complex (Chen et al. 1995). TRIP-1 association with TßRII is dependent on kinase activity of TßRII, since kinase deficient mutants did not associate with TRIP-1. Furthermore, TRIP-1 is phosphorylated by TßRII, which suggests TRIP-1 functions in TGFß signaling (Chen et al. 1995). Choy and Dernyck (1998) conducted further studies to investigate the possible function of TRIP-1. Overexpression of TRIP-1 resulted in a decrease in TGFß induced reporter gene and endogenous gene expression, indicating TRIP-1 to be an inhibitor of TGFß signaling (Choy & Derynck 1998).

The immunophilin FKBP-12 was identified as a TßRI binding protein, and inhibits TGFß signaling by preventing phosphorylation of TßRI by TßRII (Wang et al. 1996a; Chen et al. 1997). Ligand binding causes dissociation of FKBP-12 from TßRI allowing phosphorylation and activation of TßRI to proceed (Wang et al 1996a; Chen et al 1997). Chen et al. (1997) showed that mink lung epithelial cells expressing mutant FKBP-12 exhibit "leaky" signaling activity, hence, FKBP-12 may regulate TGFß signaling to prevent inappropriate TßRI phosphorylation (Chen et al. 1997). In addition, mutant versions of TßRI which are unable to bind FKBP-12 are hypersensitive to phosphorylation by TßRII. STRAP interacts with TßRI, which acts synerigistically with Smad7 to inhibit TGFß signaling (Datta et al. 1998; Datta & Moses 2000). Farnesyltransferase α associates with TßR1 in the absence of ligand, which is phosphorylated and released in response to ligand binding (Wang et al. 1996b). BAMBI is a protein related to type I receptors of the TGFß superfamily, and association of BAMBI with TßRI inhibits TGFß signaling by inhibiting TßRI and TßRII association (Onichtchouk et al. 1999). TRAP-1 can interact with ligand-activated or constitutively

active TßRI mutants, but does not bind to wild-type inactive TßRI (Charng et al. 1998). Reporter gene assays show TRAP-1 inhibits TGFß signaling, and may also function to regulate TGFß signaling (Charng et al. 1998). Association of proteins with both type I and type II receptors modulate TGFß signaling, and likely act as regulatory mechanisms to ensure proper activation of Smads and downstream signaling.

1.2.6 Smads

TGFß activation of receptors leads to signaling via intracellular signaling molecules. These signaling molecules were first described in *Drosophila*, and are activated in response to a TGFß related protein known as decapentaplegic (Dpp). In *Drosophila*, the intracellular signaling molecules are known as Mothers against Dpp (Mad). Homologous proteins were identified in *C.elegans*, and are referred to as Sma. As such, vertebrate homologs of these intracellular signaling molecules are known as Smads, for Sma/Mad (Heldin et al. 1997; Massagué 1998; Massagué & Wotton 2000; Attisano & Wrana 2002). There are 3 classes of Smads; receptor-activated Smads (R-Smad), a common-mediator Smad (Co-Smad), and inhibitory Smads (I-Smad). The R-Smads responsible for TGFß signaling are Smad2 and Smad3. TßRI phosphorylates R-Smads, and once Smad2 and Smad3 are phosphorylated, they interact with the Co-Smad, Smad4 (Nakao et al. 1997a; Macias-Silva et al. 1996; Zhang et al. 1996). I-Smads, Smad6 and Smad7, are activated by members of the TGFß superfamily, and act to negatively regulate signaling (Heldin et al. 1997; Massagué 1998; Gold 1999).

All Smads contain a Mad-homology 1 (MH1) domain, a Mad-homology 2 (MH2) domain, and a linker domain (Figure 1-3). The amino-terminal MH1 domain of R-Smads and Smad4 is responsible for DNA binding, which is essential for gene expression

(Heldin et al. 1997; Massagué & Wotton 2000). Unlike Smad3, Smad2 contains an insert within the MH1 domain that causes Smad2 to be unable to bind DNA (Dennler et al. 1998; Shi et al. 1998; Zawel et al. 1998). All Smads share a similar MH2 domain, which is responsible for Smad interaction with other proteins, receptor recognition, and Smad oligomerization (Heldin et al. 1997; Wu et al. 1997; Massagué 1998; Massagué & Wotton 2000). The MH2 domain of R-Smads contains a carboxy terminal SSXS motif. The last 2 serines of this motif are phosphorylated by TßRI which is necessary for signaling (Macias-Silva et al. 1996; Nakao et al. 1997a; Souchelnytskyi et al. 1997). The MH2 domain of R-Smads also contains the L3 loop which is responsible for substrate specificity, and associates with the L45 loop of the type I receptor (Lo et al. 1998). Autoinhibition of the Smads occurs by association of the MH1 and MH2 domains (Hata et al. 1997). Upon activation by the type I receptor, the autoinhibition is released and the R-Smads can associate with Smad4, and this Smad4/R-Smad interaction is necessary for signaling (Lagna et al. 1996; Abdollah et al. 1997; Nakao et al. 1997a; Souchelnytskyi et al. 1997). The elucidation of the heteromeric Smad complexes that form in response to TGFß signaling has been difficult and remains controversial. Inman et al. (2002) examined Smad complex formation, and their data suggests that the Smad2 heteromeric complex consists of two Smad2 proteins and one Smad4, whereas the Smad3 complex contains one Smad3 and one Smad4 (Inman & Hill 2002).

Smad6 and Smad7 are I-Smads. Smad6 is responsible for negative regulation of the BMP pathway, whereas Smad7 is involved in negative regulation of TGFß signaling by association with the active receptor complex and interfering with R-Smad phosphorylation (Hayashi et al. 1997; Nakao et al. 1997b). TGFß enhances Smad7

expression, causing negative feedback inhibition (Nakao et al. 1997b). Smad7 is primarily nuclear in the absence of ligand, and upon TGFß signaling activation, Smad7 becomes predominantly cytoplasmic (Itoh et al. 1998). A domain within the MH2 domain of Smad7 is required for nuclear localization and TGFß induced export to the cytoplasm, since deletion mutations within the MH2 domain produced exclusive cytoplasmic localization of Smad7 (Itoh et al. 1998).

1.2.7 Smad nuclear shuttling

Once the R-Smads are phosphorylated, they interact with Smad4 and translocate to the nucleus to modulate target gene expression (Figure 1-4). The mechanisms involved in nuclear import are diverse for each Smad. Smad4 continuously shuttles between the nucleus and cytoplasm when the TGFß signaling pathway is not activated by ligand (Pierreux et al. 2000). A constitutively active nuclear export signal (NES) and a constitutively active nuclear localization signal (NLS) are responsible for the continuous shuttling (Inman et al. 2002). The NES is exportin-dependent and is located in the linker region, whereas the NLS is importin-dependent and localized to the MH1 domain (Pierreux et al. 2000). Pierreux et al. (2002) suggest that upon activation of TGFß signaling, association of Smad2 or Smad3 with Smad4 alters the NES, causing Smad4 to be retained within the nucleus. Smad3 contains a NLS within the MH2 domain, which if mutated, results in inhibition of Smad3 nuclear translocation. Xiao et al. (2000a) proposed that this NLS is masked by the MH1 domain in the autoinhibited Smad3, and once phosphorylation occurs by TßRI, the conformational change causes the NLS to be unmasked and nuclear localization to occur (Xiao et al. 2000a). Nuclear localization of Smad3 is importin-ß/ran-dependent (Xiao et al. 2000b; Kurisaki et al. 2001).

In contrast, similar studies have found that Smad2 nuclear import is ran-independent. Although a similar NLS sequence occurs in the MH1 domain of Smad2, the insertions within the MH1 domain of Smad2 interferes with the NLS, and therefore, Smad2 enters the nucleus by an alternative pathway (Kurisaki et al. 2001). Two inserts occur within the MH1 domain of Smad2, termed GAG and TID, and it was determined by Kurisaki et al. (2001) that the TID insert prevents association of Smad2 with importin-ß (Kurisaki et al. 2001). Smad2 directly associates with the nucleoporins CAN/nup214 and Nup513 on the cytoplasmic and nuclear side of the nuclear pore, respectively (Xu et al. 2002). Association of a hydrophobic region in the MH2 domain of Smad2 mediates this interaction, which induces nuclear import or export (Xu et al. 2002). Nuclear import of Smad2 has been shown to be inhibited by Smad anchor for receptor activation (SARA; Tsukazaki et al. 1998). Smad2 associates with SARA, which is a FYVE domain protein and recruits Smad2 to the TGFß type I receptor for activation. Mutation of the FYVE domain of SARA causes disruption in Smad2 localization and inhibition of TGFß signaling (Tsukazaki et al. 1998). Once Smad2 is phosphorylated by the type I receptor, the SARA/Smad2 complex dissociates, and Smad2 associates with Smad4 and is translocated to the nucleus (Xu et al. 2000). Smads are de-phosphorylated in the nucleus, which causes export to the cytoplasm, and has been shown to be exportin-independent (Inman et al. 2002). Provided the receptors are still active, the Smads are re-phosphorylated and signaling continues (Inman et al. 2002).

1.2.8 Smad transcriptional responses

Within the nucleus, Smads act as transcription factors and associate with other transcription factors to modulate target gene expression. Smad3 and Smad4 bind a

specific DNA sequence, CAGAC, known as a Smad-binding element (SBE) (Shi et al. 1998; Zawel et al. 1998). Efficient transcription of TGFß target genes occurs by Smad complex binding to the SBE, in association with other transcription factors binding to their specific sequences at the promoters of target genes (Heldin et al. 1997; Massagué 1998; Massagué & Wotton 2000). Smad2 and Smad3 associate with a number of transcription factors, including AP-1, ATF2, TFE3, Sp1, MIZ-1, and FAST (Labbé et al. 1998; Zhang et al. 1998; Zhou et al. 1998; Hua et al. 1998; Sano et al. 1999; Wong C et al. 1999; Feng et al. 2000; Seone et al. 2001). R-Smads also interact with the co-activator, CBP/p300, which has histone acetyltransferase activity and can therefore activate transcription by altering chromatin structure (Feng et al. 1998; Janknecht et al. 1998; Pouponnot et al. 1998; Shen et al. 1998). Smad association with the different transcription factors determine which TGFß target gene will be modulated by signaling activity.

1.2.9 Smad association with repressors

In addition to association with transcription factors, Smads also interact with repressors resulting in inhibition of TGFß-induced transcription. Ski is a transcriptional repressor that antagonizes TGFß signaling (Akiyoshi et al. 1999; Luo et al. 1999; Sun et al. 1999). Ski has been shown to associate with Smad2 (Akiyoshi et al. 1999; Luo et al. 1999), Smad3 (Akiyoshi et al. 1999; Luo et al. 1999; Sun et al. 1999), and Smad4 (Luo et al. 1999) in a ligand-dependent manner. TGFß-responsive promoter elements are repressed by Ski association with the Smad complex at the promoter, via recruitment of the nuclear co-repressor, N-CoR (Luo et al. 1999), and association of histone deacetylase 1 (HDAC1) (Akiyoshi et al. 1999). Furthermore, overexpression of Ski causes resistance

to the anti-proliferative effect of TGFß (Luo et al. 1999, Sun et al. 1999). Ski alone does not bind DNA, but requires association with Smads to bind DNA at SBE's to repress TGFß transcriptional responses (Luo et al. 1999). SnoN, a transcriptional repressor related to Ski, interacts with Smad2, Smad4, and to a lesser extent, Smad3, resulting in TGFß transcriptional repression by recruitment of N-CoR (Stroschein et al. 1999).

TGIF, a transcriptional co-repressor, associates with Smad2 causing TGIF to be recruited to TGFß target genes to inhibit transcription (Wotton et al. 1999a; Wotton et al. 1999b). TGIF caused inhibition of transfected TGFß target genes in mink lung epithelial cells, and inhibition of TGIF by antisense resulted in an increase in endogenous TGFß target gene expression (Wotton et al. 1999a). Wotton et al. (1999a) also determined that in response to TGFß, a protein complex containing TGIF, Smad2, Smad4, the transcription factor FAST2, and HDAC1 formed. They further determined that TGIF association was dependent on Smad2, and HDAC association was dependent on TGIF (Wotton et al. 1999a). The recruitment of HDAC likely induces the transcriptional repression, and the authors propose a model in which TGIF competes with transactivators for binding with Smad2 (Wotton et al. 1999a). Hence, within the nucleus competition for Smad binding occurs between co-activators and co-repressors, with the outcome dependent on levels of co-activators or co-repressors (Wotton et al. 1999a), thus adding to the regulation of TGFß signaling activity. A further study by the same group found transcriptional repression of TGFß target genes by TGIF is mediated by different repression domains of TGIF (Wotton et al. 1999b). Mutational analysis reveals that partial repression can still occur when the region of TGIF that interacts with HDAC is deleted. Although partial repression can occur independent of HDAC, the repressive

effects of TGIF are highly dependent on HDAC activity (Wotton et al. 1999b). TGIF has also been shown to interact with the transcriptional co-repressor mSin3 in response to TGFß (Wotton et al. 2001), which may contribute to HDAC association and repression of TGFß transcription. Another transcriptional co-repressor related to TGIF, TGIF2, has been identified (Imoto et al. 2000; Melhuish et al. 2001) that also interacts with Smads to inhibit TGFß transcriptional responses (Melhuish et al. 2001). In addition, TGIF2 is amplified and overexpressed in ovarian cancer cell lines, thereby suggesting a role for TGIF in ovarian tumorigenesis, although this has not yet been investigated (Imoto et al. 2001). Smad association with transcriptional repressors likely serves to regulate TGFß signaling.

1.2.10 Smad degradation

Following transcriptional activation of TGFß target genes by Smads, degradation of the Smads may occur. Smads are degraded by ubiquitination, a process that adds ubiquitin to proteins by a series of enzymatic steps. Ubiquitin-activating enzyme (E1) activates ubiquitin in an ATP-dependent mechanism. Ubiquitin is then transferred to ubiquitin-conjugating enzyme (E2), which may require the activity of ubiquitin ligase (E3) resulting in ubiquitin linked to the target protein. Multiple ubiquitin proteins are conjugated to the target protein, which is recognized by a complex of proteases known as the 26S proteosome, resulting in protein degradation (Yamao 1999). Smad ubiquitination regulatory factor (Smurf) is an E3-type, Hect-domain ubiquitin ligase that interacts with Smads to target them for degradation (Lin et al. 2000). Smurf1 has been shown to target the BMP Smad, Smad1, for degradation (Zhu et al. 1999), whereas Smurf2 interacts with Smad2, as well as the Smad1 (Lin et al. 2000; Zhang et al. 2001). Although Smurf2 can

interact with Smad3, Smurf2 does not mediate Smad3 degradation (Zhang et al. 2001). Smad3 degradation also occurs by ubiquitination, however, SCF/Roc1 E3 ligase mediates Smad3 ubiquitination (Fukuchi et al. 2001). Fukuchi et al. (2001) also demonstrate that Smad2 is not degraded by the same SCF/Roc1 ubiquitin ligase complex, hence, degradation of the Smads occur by independent mechanisms. Interestingly, Smad3 is degraded in the cytoplasm, following transcriptional activation. Hence, the ubiquitin ligase may contribute to nuclear export of Smad3, since the mechanism of Smad nuclear export is yet unidentified (Fukuchi et al. 2001). Smad4 degradation occurs by ubiquitination by Jab1, followed by degradation by the 26S proteosome (Wan et al. 2002). In addition, Xu and Attisano (2000) describe ubiquitination of mutant Smad2 and Smad4. A missense mutation in the MH1 domain of Smad2 and Smad4 occurs in colorectal and pancreatic cancers, which induces rapid degradation by induction of ubiquitination by UbcH5 ubiquitin ligase (Xu & Attisano 2000).

In addition to Smad degradation, Smurf2 has additional roles in regulation of TGFß signaling. Smurf2 binds Smad7 constitutively, which is targeted to TGFß receptors following ligand activation to degrade TGFß receptors (Kavsak et al. 2000). In addition, Smurf2 associates with SnoN in a Smad2 dependent manner (Bonni et al. 2001). Association of Smurf2 with SnoN causes degradation of SnoN, leading to enhancement and regulation of TGFß signaling (Bonni et al. 2001).

1.2.11 TGFß regulation of cell cycle progression

TGFß signaling modulates expression of a number of target genes that play roles in various cellular processes. Regulation of cellular proliferation by TGFß occurs by modulation of cell cycle regulatory genes (Figure 1-5), such as $p15^{INK4B}$ (Hannon &

Beach 1994), $p21^{CIP1}$ (Datto et al. 1995a; Li CY et al. 1995), $p27^{KIP}$ (Polyak et al. 1994a; Polyak et al. 1994b; Reynisdottir et al. 1995), and myc (Coffey et al. 1998). $p15^{INK4B}$, $p21^{CIP1}$, and $p27^{KIP}$ are cyclin-dependent kinase inhibitors (CKI) which associate with specific cyclin-dependent kinases (Cdk) to inhibit their kinase activity. This results in inhibition of phosphorylation and subsequent activation of proteins necessary for cell cycle progression (Sherr 1994). TGFß induces up-regulation of $p15^{INK4B}$ expression (Hannon & Beach 1994), which is mediated by association of the transcription factor Sp1 at Sp1 binding sites on the $p15^{INK4B}$ promoter (Li JM et al. 1995). Feng et al. (2000) describe that Smad2, Smad3 and Smad4 complex with Sp1 to induce transcription of $p15^{INK4B}$ in response to TGFß (Feng et al. 2000).

Similarly, $p21^{CIP1}$ expression is up-regulated by TGFß treatment (Elbendary et al. 1994; Datto et al. 1995a). Up-regulation of $p21^{CIP1}$ causes inhibition of Cdk4/6 and Cdk2, which results in growth inhibition (Datto et al. 1995a; Li CY et al. 1995). p53 also up-regulates $p21^{CIP1}$ expression, however, the TGFß induced up-regulation of $p21^{CIP1}$ is p53-independent (Datto et al. 1995a). TGFß-induced up-regulation of $p21^{CIP1}$ occurs similar to that of $p15^{INK4B}$, by association of Smads and Sp1 at the $p21^{CIP1}$ promoter (Datto et al. 1995b).

The effect of TGFß on $p27^{KIP}$ expression is unlike that of $p15^{INK4B}$ or $p21^{CIP1}$. The CKI, $p27^{KIP}$, is not up-regulated in response to TGFß, but the association of $p27^{KIP}$ with Cdk2 is substantially increased by TGFß treatment in mink lung epithelial cells (Reynisdottir et al. 1995). Human keratinocytes (HaCaT) cells also have enhanced $p27^{KIP}$ -Cdk2 complex formation, but this complex formation only occurred after 24h following TGFß treatment, as opposed to only 4-8h in Mv1Lu cells. Additionally,

HaCaT cells increase $p27^{KIP}$ levels in response to TGFß (Reynisdottir et al. 1995). The increase in $p27^{KIP}$ -Cdk2 complex formation in response to TGFß was correlated with a loss of $p27^{KIP}$ -Cdk4 and $p27^{KIP}$ -Cdk6 complex formation, thereby freeing Cdk4/6 for association and inhibition by $p15^{INK4B}$ (Reynisdottir et al. 1995). $p15^{INK4B}$ expression itself can cause a decrease in $p27^{KIP}$ association with Cdk4/6, and enhance $p27^{KIP}$ -Cdk2 formation (Reynisdottir et al. 1995).

Myc is a protein that can either activate or repress transcription, depending on its associated factors (Facchini and Penn 1998; Dang 1999). A related protein, max, associates with myc to activate target genes (Staller et al. 2001). TGFß down-regulates expression of myc, thereby causing inhibition of cell proliferation (Pietenpol et al. 1990; Zentella et al. 1991). Warner et al. (1999) found that forced expression of myc inhibits the TGFß induced up-regulation of $p15^{INK4B}$ expression and suggest this inhibition may be due to myc repression at the $p15^{INK4B}$ promoter (Warner et al. 1999). Indeed, Staller et al. (2001) found that in the absence of TGFß, myc and max are associated with miz-1 at the $p15^{INK4B}$ promoter. Miz up-regulates $p15^{INK4B}$ expression, however the presence of myc and max block miz activity causing inhibition of $p15^{INK4B}$ expression allowing cell cycle progression to occur. TGFß causes down-regulation of myc, thereby releasing miz-1 and $p15^{INK4B}$ transcription is up-regulated resulting in cell cycle arrest (Staller et al. 2001). Furthermore, Seoane et al. (2001) show that both Smad transactivation and down-regulation of myc are required to control $p15^{INK4B}$ expression in response to TGFß (Seoane et al. 2001). Recently, Feng et al. (2002) show that myc can interact directly with Smad2 and Smad3 in HaCaT cells. Overexpression of myc repressed TGFß-induced $p15^{INK4B}$ expression in mink lung epithelial cells (Feng et al. 2002). Myc binds

to the Smad and Sp1 complex at the $p15^{INK4B}$ promoter to inhibit TGFß-induced $p15^{INK4B}$ expression (Feng et al. 2002). Clearly, regulation of cell proliferation by TGFß involves a number of cell cycle regulatory genes that are modulated by TGFß.

1.2.12 Other TGFß target genes

Additional target genes modulated by TGFß play roles in various cellular processes, contributing to the diversity of TGFß cellular effects. TGFß can induce transcription of a number of genes that contain TPA-response elements (TRE), such as collagenase I, by association of Smads with AP-1 (Zhang et al. 1998; Wong C et al. 1999). TGFß functions in ECM stabilization by inducing expression of protease inhibitors, such as plasminogen activator inhibitor type I (PAI-1) (Lund et al. 1987; Keski-Oja et al. 1988a). PAI-1 inhibits both tissue type plasminogen activator (tPA) and urokinase plasminogen activator (uPA), and multiple TGFß-responsive elements were suggested to play a role in TGFß induced expression of PAI-1 (Westerhausen et al. 1991). Indeed, Smad interaction with the transcription factor TFE (Hua et al. 1998), and the Smad-Sp1 interaction (Datta et al. 2000) play a role in up-regulation of PAI-1 expression in response to TGFß.

Smad7 expression is up-regulated in response to TGFß which participates in negative feedback inhibition of signaling (Nakao et al. 1997b). Smad3 and Smad4 are necessary for TGFß induced up-regulation of Smad7 (von Gersdorff et al. 2000). TFE association with a Smad3 complex is also necessary for TGFß induced Smad7 expression (Hua et al. 2000). Additional TGFß target genes exist, and TGFß target gene expression depends on interaction of Smads with specific transcription factors to appropriately regulate transcription.

1.2.13 TGFß signaling crosstalk

TGFß signaling can be modulated by other signaling pathways that converge with the TGFß signaling pathway to alter its effects. Depending on the cell context, interferon gamma (IFNγ), hepatocyte growth factor (HGF), epidermal growth factor (EGF) and oncogenic ras have all been shown to modulate TGFß signaling (de Caestecker et al. 1998; Kretzschmar et al. 1999; Ulloa et al. 1999; ten Dijke et al. 2000; Funaba et al. 2002).

Enhanced TGFß signaling was demonstrated in studies by de Caestecker et al. (1998) and Funaba et al. (2002). HGF induced phosphorylation of Smad2 increased Smad nuclear translocation in mink lung epithelial cells (Mv1Lu), and it is suggested that EGF would have a similar effect (de Caestecker et al 1998). The site of Smad2 phosphorylation in response to HGF was found to be at the same site which TGFß phosphorylates at the carboxy terminal domain. This may indicate that HGF can enhance TGFß signaling, although the kinase responsible for HGF phosphorylation is not TßRI (de Caestecker et al. 1998). Funaba et al. (2002) also show enhanced TGFß signaling in COS-7 and Mv1Lu cells as a result of EGF-induced phosphorylation of Smad2, which is mediated by Erk1. The phosphorylation site was found to be in the amino terminal domain of Smad2, and it was further determined that this phosphorylation event extended the half-life of Smad2, which would thereby enhance Smad2 mediated responses (Funaba et al. 2002). These studies suggest that signaling via Erk kinases can enhance TGFß signaling.

In contrast to the studies demonstrating enhanced TGFß signaling by MAPK signaling, inhibition of TGFß signaling by Ras signaling has been described. Ras-transformed murine mammary epithelial cells demonstrate inhibition of the TGFß signaling pathway, and these cells are resistant to the anti-proliferative effect of TGFß (Kretzschmar et al. 1999). TGFß-induced Smad2 and Smad3 nuclear translocation was inhibited by oncogenic ras, which likely explains the inhibition of TGFß cellular response by Ras in murine mammary epithelial cells (Kretzschmar et al. 1999). The inhibition of Smad nuclear translocation by Ras and EGF was due to phosphorylation of residues in the linker region of Smad2 and Smad3 by EGF (Kretzschmar et al. 1999).

IFNγ has also been shown to inhibit TGFß signaling, however, the mechanism of TGFß signaling inhibition by IFNγ involves a different stage in the TGFß signaling pathway. Smad3-Smad4 complex formation and subsequent nuclear translocation are inhibited by IFNγ in a human fibrosarcoma cell line (Ulloa et al. 1999). IFNγ up-regulates Smad7, which acts to negatively regulate the TGFß signaling pathway. Furthermore, the IFN-γ induced up-regulation of Smad7 blocked Smad3-TßRI complex formation and Smad3 phosphorylation (Ulloa et al. 1999). Therefore, Smad7 induction by alternative signaling pathways may contribute to TGFß signaling inhibition.

TGFß signaling has also been shown to be inhibited by androgen and estrogen (Evangelou et al. 2000; Matsuda et al. 2001; Chipuk et al. 2002). 5α-dihydrotestosterone (DHT) down-regulates TGFß receptors in ovarian cancer cell lines, which may inhibit TGFß signaling (Evangelou et al. 2000). Chipuk et al. (2002) found that the androgen receptor inhibits Smad3 interaction with SBE, thereby inhibiting TGFß signaling in a prostate cancer cell line (Chipuk et al. 2002). Similarly, the estrogen receptor was found

to associate with Smad3 in kidney carcinoma cells to inhibit TGFß signaling (Matsuda et al. 2001).

Studies examining factors that can affect TGFß signaling show both enhancement and inhibition of TGFß signaling, and the effect of these other signaling pathways on TGFß signaling is likely cell type dependent. Clearly, numerous factors involved in other signaling pathways may act to modulate the various stages of the TGFß signaling pathway, which may contribute to the altered response to TGFß typically seen in cancer.

1.2.14 TGFß in cancer

TGFß is a bifunctional protein which plays a role in normal tissue homeostasis via its anti-proliferative role, which is also the role TGFß plays early in tumorigenesis. During tumor progression, however, factors produced by cancer cells likely cause TGFß to switch from being a tumor suppressor, to function as a tumor promoter (Akhurst 2002). Late in tumor progression TGFß plays a role in tumorigenesis by promoting angiogenesis, immunosuppression, and metastasis (Oft et al. 1998; Yu and Stamenkovic 2000).

The proliferative and metastatic properties of TGFß are affected by ras activation. Non-metastatic transformed murine fibroblasts are growth inhibited by TGFß, whereas metastatic murine fibroblasts transformed with H-ras are growth stimulated by TGFß (Schwarz et al. 1988). Similarly, TGFß induced invasive properties in ras-transformed murine mammary epithelial cells, but not non-transformed normal cells (Oft et al. 1996). Furthermore, Janda et al. (2002) demonstrate that TGFß-induced metastasis is dependent on oncogenic ras. Therefore, oncogenic ras appears to be required for the tumor promoting properties of TGFß.

TGFß signaling defects commonly occur in cancer, resulting in resistance to the anti-proliferative effect of TGFß. Mutations in receptors and Smads have been implicated in pancreatic, colorectal, lung, and gastric cancers. TßRI mutations have been described in chronic lymphocytic leukemia (CLL), prostate, breast, cervical and gastric cancer (Kim et al. 1996; DeCoteau et al. 1997; Chen et al. 1998; Chen et al. 1999; Kang et al. 1999). One-third of CLL patients are resistant to the anti-proliferative effect of TGFß, and thus were examined for TGFß receptor mutations. In contrast to primary CLL cells from patients that are sensitive to the anti-proliferative effect of TGFß, cells from TGFß resistant patients show loss of expression of TßRI protein, thereby suggesting type I receptor loss to be the mechanism of TGFß resistance (DeCoteau et al. 1997). The TGFß resistant prostate cancer cell line LNCaP was found to have loss of expression of TßRI mRNA and protein, and demonstrate a genetic alteration in the TßRI gene. Sensitivity to TGFß was restored by addition of TßRI to these cells by transient transfection (Kim et al. 1996). Breast cancer cell lines are typically resistant to the anti-proliferative effect of TGFß, and in one study 2/31 primary breast cancers and 5/21 lymph node metastases had a TßRI receptor kinase mutation, which resulted in loss of signaling activity (Chen et al. 1998). Primary cervical cancer samples were examined for both type I and type II receptor mutations, and 13/16 samples either exhibited a polymorphism or a deletion in TßRI, which may contribute to the decreased responsiveness to TGFß (Chen et al. 1999). Hypermethylation of TßRI was found to be the cause of decreased TßRI expression in gastric cancer cell lines and primary gastric tumors (Kang et al. 1999). Hence, TßRI mutations may contribute to resistance to the anti-proliferative effect of TGFß in some cancers.

TßRII receptor mutations are more common than TßRI receptor mutations, due to the presence of repeated sequences that are susceptible to mutation (Markowitz et al. 1995), and have been described in lung, gastric, and colon cancers (Park et al. 1994; Markowitz et al. 1995; Myeroff et al. 1995; Osada et al. 2001). Markowitz et al. (1995) found TßRII mutations in colon cancer cell lines and decreased or loss of TßRII expression was found, which correlated with loss of TGFß binding (Markowitz et al. 1995). Further characterization of these mutations revealed all cell lines with reduced or loss of TßRII expression carried the same mutation, which is associated with colon cancers exhibiting microsatellite instability (Markowitz et al. 1995). These TßRII mutations were later identified in gastric cancers as well (Myeroff et al. 1995). A previous study had found TßRII receptor mutations in gastric cancer cell lines that were resistant to the anti-proliferative effect of TGFß (Park et al. 1994). The mechanism of loss of TßRII expression in lung cancer cell lines is different than that of colon and gastric cancers. Lung cancers typically do not exhibit microsatellite instability, therefore, this inactivating mutation is unlikely. Osada et al. (2001) found that loss of TßRII expression in lung cancer cell lines occurred due to epigenetic mechanisms; specifically, enhanced methylation of DNA within the TßRII promoter region and histone deacetylation (Osada et al. 2001).

Smad mutations have also been implicated in cancer. Smad4 was originally identified as DPC4 (deleted in pancreatic carcinoma), which is deleted in the majority of pancreatic cancers (Hahn et al. 1996). The function of DPC4 was not known at this time, but was identified as a tumor suppressor (Thiagalingam et al. 1996) and suggested to play a role in TGFß signaling. DPC4 was later found to be mutated in lung cancers (Nagatake

et al. 1996). A subsequent study demonstrated that DPC4 mutations or deletions were not a common event in other types of cancers (Schutte et al. 1996), however, Takagi et al. (1996) demonstrated that DPC4 was also mutated in colorectal cancer (Takagi et al. 1996). These studies also suggested that another tumor suppressor must be involved, due to the fact that allelic loss at 18q21 was often seen in these cancers, however, DPC4 mutations did not occur in all cases (Nagatake et al. 1996; Takagi et al. 1996). Indeed, Smad2 is also located at chromosome 18q21, and has also been implicated as a tumor suppressor (Eppert et al. 1996; Riggins et al. 1997). Eppert et al. (1999) found inactivating mutations in Smad2, then known as MADR2, in colorectal cancer (Eppert et al. 1999), and Smad2 mutations were also identified in lung cancer (Uchida et al. 1996; Riggins et al. 1997). Although Smad3 mutations have not been described, Zhu et al. describe a Smad3 knockout mouse which develops metastatic colorectal cancer (Zhu et al. 1998), although tumor formation has not been reported in other Smad3 null mice (Ashcroft et al. 1999; Datto et al. 1999; Yang et al. 1999). Mutations in components of the TGFß signaling pathway clearly play a role in tumorigenesis in a variety of cancers.

1.2.15 TGFß in ovarian cancer

Due to the role that TGFß signaling defects play in other cancer types, TGFß signaling mutations and altered responses to TGFß cellular effects have been studied in ovarian cancer. Henriksen et al. (1995) found TGFß ligand was overexpressed in ovarian tumor samples compared to normal ovarian epithelial cells, however, no change in the levels of TßRI or TßRII was found (Henriksen et al. 1995). Similarly, another study also found increased expression of TGFß ligand, however, reduced expression of TßRI was found in ovarian cancer samples compared to normal ovarian tissue (Bristow et al. 1999).

Chen et al. (2001) also examined TßRI in ovarian cancer samples, and found 33% of ovarian cancers have TßRI mutations (Chen et al. 2001). TßRII mutations have also been identified. Decreased or loss of TßRII expression was found in 15/22 ovarian tumor samples (Lynch et al. 1998). An independent study also found TßRII mutations, which were significantly correlated with histology (Francis-Thickpenny et al. 2001). The majority of TßRII mutations occurred in clear cell carcinomas (Francis-Thickpenny et al. 2001). An additional study examined mutations in TßRI, TßRII, Smad2 and Smad4 in ovarian tumor samples, and found 65.6% harbored a mutation in one of these components of the TGFß signaling pathway (Wang et al. 2000). Mutations in TßRI, TßRII, and Smad2 were identified in this study, however, no Smad4 mutations were detected.

Proliferation of primary ovarian cancer cells and ovarian cancer cell lines in response to TGFß has been investigated, considering resistance to the anti-proliferative effect of TGFß is a common occurrence in other cancer types. Ovarian cancer cell lines have been found to be resistant to the anti-proliferative effect of TGFß. Berchuck et al. (1992) examined the anti-proliferative effect of TGFß in normal ovarian epithelial cells and ovarian cancer cell lines. Using [^{3}H] thymidine incorporation assays to measure proliferation, normal OSE was found to be sensitive to the anti-proliferative effect of TGFß (Berchuck et al. 1992). Although one ovarian cancer cell line (OVCA420) was found to respond to the anti-proliferative effect of TGFß, 4 other cell lines were either unaffected or showed only a slight decrease in thymidine incorporation in response to TGFß (Berchuck et al. 1992). The effect of TGFß on primary ovarian cancer cells was not assessed in this study. The ovarian cancer cell lines CaOV3 and OVCAR3 were found to be growth inhibited by TGFß using MTT assays (Zhou and Leung 1992).

Hence, contradictory reports exist in the literature regarding the effect of TGFß on proliferation of ovarian cancer cell lines. Additionally, the reports investigating the anti-proliferative effect of primary ovarian cancer cells are also contradictory. Yamada et al. (1999) measured proliferation using a MTT assay with primary ovarian cancer cells isolated from ovarian tumor explants. The majority of primary ovarian cancer cells (78%) were resistant to the anti-proliferative effect of TGFß (Yamada et al. 1999). In contrast, Hurteau et al. (1994) found 95% of primary ovarian cancer cells to be sensitive to the anti-proliferative effect of TGFß. This study measured proliferation using [^{3}H]-thymidine incorporation, and used primary ovarian cancer cells isolated from ascites fluid (Hurteau et al. 1994). Assay method or source of tumor cells may be the cause of these contradictory results.

Clearly, the role of TGFß signaling in ovarian cancer remains controversial. Contradictory results regarding TGFß receptor expression and mutations, as well as conflicting studies examining the response of ovarian cancer cells to the anti-proliferative effect of TGFß elicit the need to further examine this pathway to define its role in ovarian tumorigenesis.

1.3 Hypothesis and Objective

Although studies exist that examine the TGFß signaling pathway and TGFß cellular responses in ovarian cancer cells, the results are contradictory. Considering other types of cancers exhibit defects in the TGFß signaling pathway and are resistant to the anti-proliferative effect of TGFß, it is likely that TGFß signaling defects occur in ovarian cancer and that ovarian cancer cells do not respond to the growth inhibitory effect of TGFß. The growth inhibitory effect of TGFß could be modulated in a number of ways. Mutations in TGFß receptors or Smads could cause the TGFß signaling pathway to be non-functional. Similarly, defects in TGFß target gene expression could occur to inhibit cellular responses to TGFß. Furthermore, the presence of other growth factors could inhibit TGFß signaling by modulating various steps within the TGFß signaling pathway.

Clearly, ovarian cancer cells *in vivo* are resistant to the anti-proliferative effect of TGFß considering they continue to proliferate. My hypothesis is that primary ovarian cancer cells contain defects within the TGFß signaling pathway, allowing uncontrolled cellular proliferation. The primary objective of this study was to determine whether the TGFß signaling pathway was intact and functional in primary ovarian cancer cells isolated from ascites fluid and grown in monolayer culture, and whether primary ovarian cancer cells respond to the anti-proliferative effect of TGFß. Either TGFß signaling defects or modulation by other factors could cause resistance to the growth inhibitory effect of TGFß, and these possibilities were explored in this study. A possible mechanism by which ovarian cancer cells could be resistant to the anti-proliferative effect of TGFß *in vivo* is suggested.

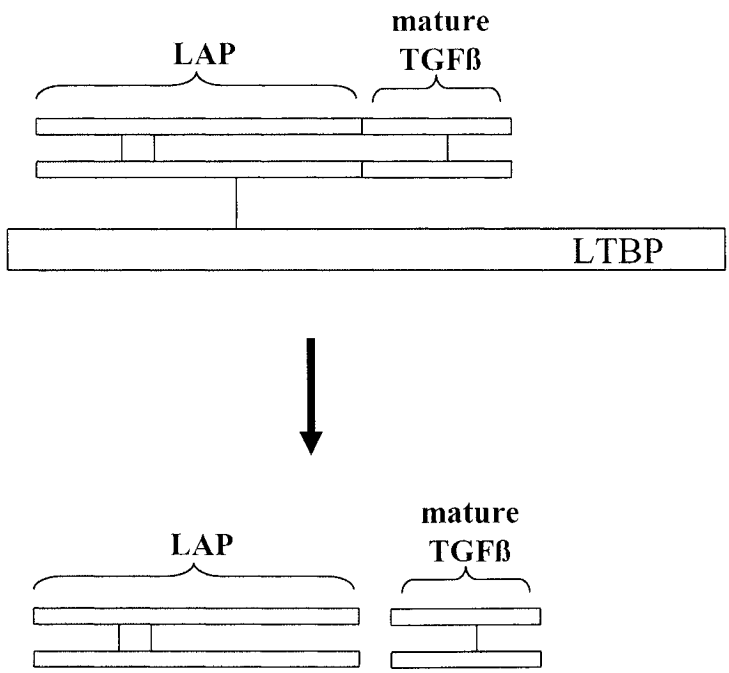


Figure 1-1: Latent TGFß structure. Latent TGFß is secreted as an inactive precurser. Latent TGFß binding protein (LTBP) functions to target latent TGFß to the ECM. Following secretion, mature TGFß remains non-covalently associated with latency associated peptide (LAP), which may remain associated with LTBP. Activation of TGFß by proteases or TSP-1 releases mature TGFß, which is now able to bind to the TGFß receptor.

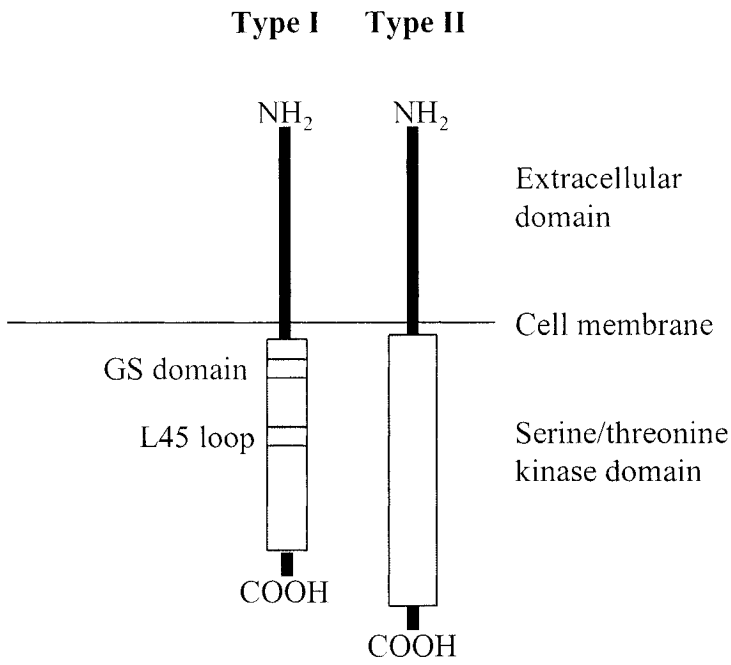
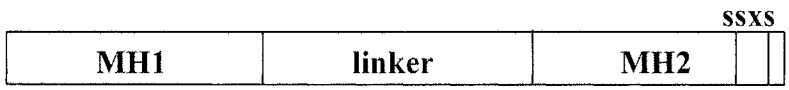


Figure 1-2: TGFß receptor subtypes. Type I and type II receptor subtypes are serine/threonine transmembrane kinase receptors. Both type I and type II subtypes have an extracellular domain, a transmembrane domain, and a serine threonine kinase domain. The TGFß type I receptor contains the GS domain which is the site of phosphorylation by the type II receptor, and the L45 loop, which is the site of association with the receptor-activated Smads. The type II receptor is constitutively phosphorylated.

A.



B.

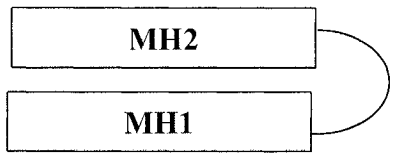


Figure 1-3: Smad functional domains. A) All Smads have an amino-terminal MH1 domain, a linker domain and a carboxy-terminal MH2 domain. The MH1 domain contains the DNA binding domain, and interacts with CAGAC DNA sequences. The linker domain contains residues that can be phosphorylated by MAP Kinases. Protein interactions with other Smads and transcription factors occur via regions within the MH2 domain. The MH2 domain also contains the L3 loop, which interacts with the L45 loop of the type I receptor. The last 2 serines within the SSXS motif are phosphorylated by the type I receptor. **B)** Autoinhibition of receptor-activated Smads occur by interaction of the MH1 and MH2 domains. Once the SSXS domain is phosphorylated by the Type I receptor, this autoinhibition is released, and the R-Smads can interact with the Co-Smad.

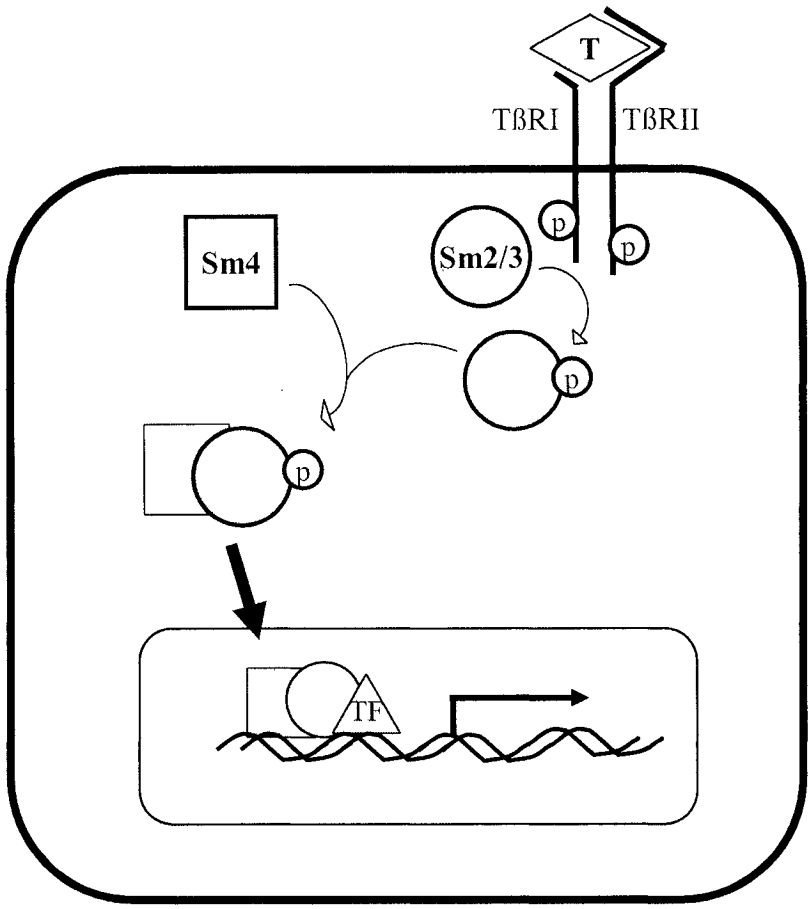


Figure 1-4: The TGFß signaling pathway. A TGFß dimer (T) binds to a dimer of TßRII, which is constitutively phosphorylated. Binding causes recruitment of TßRI, which is phosphorylated (p) by TßRII. A Smad2/3 (Sm2/3) homo or heterodimer is then phosphorylated by the type I receptor. The activated Smad hetero/homodimer then associates with Smad4 (Sm4), and this heteromeric Smad complex translocates to the nucleus. In the nucleus, the Smad complex interacts with other transcription factors (TF) to modulate TGFß target gene expression.

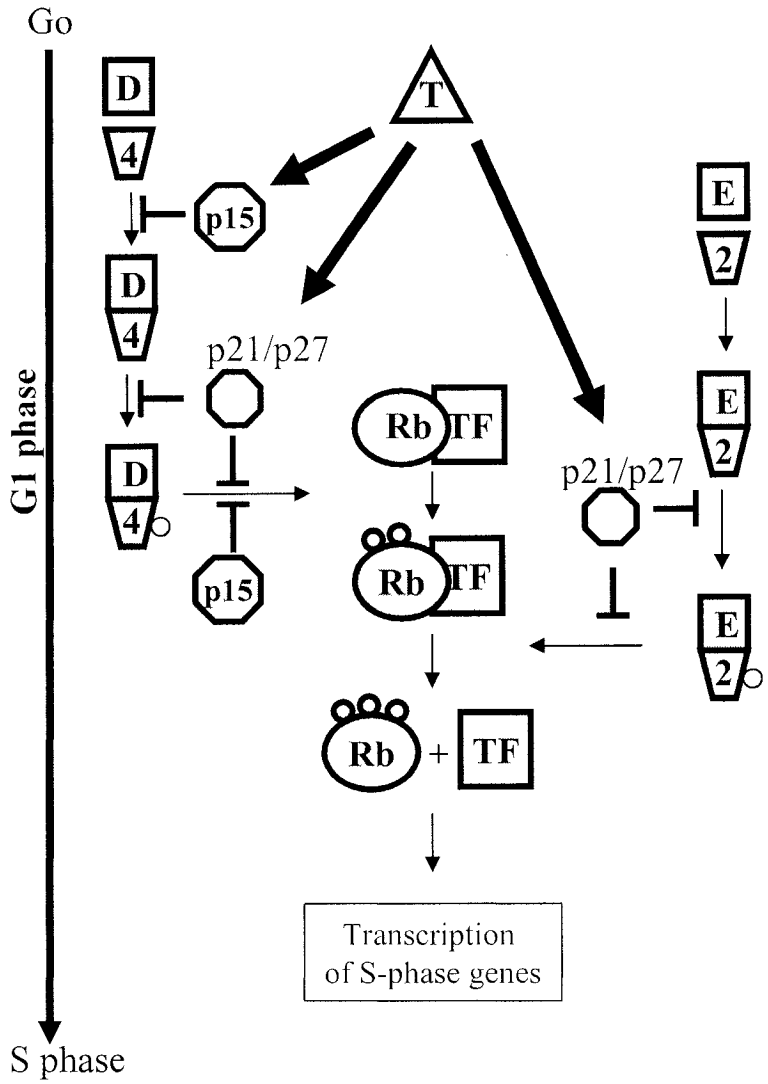


Figure 1-5: TGFß effects on cell cycle regulators in G1 phase of the cell cycle. Progression through G1 phase of the cell cycle occur via a series of cdk/cyclin interactions. Cyclins associate with Cdk's to form an active kinase which then phosphorylates retinoblastoma (Rb), which causes release of a transcription factor (TF) necessary for transcription of S-phase genes. Cyclin D (D) associates with cdk4/6 (4) in early G1, and cyclin E (E) associates with cdk2 later in G1 phase. TGFß (T) induces expression of $p15^{INK4B}$, $p21^{CIP1}$, and $p27^{KIP1}$, which act to inhibit kinase activity of cdk's. This results in inhibition of Rb phosphorylation and G1 cell cycle arrest.

CHAPTER 2

MATERIALS AND METHODS

2.1 Primary human ovarian cancer cells

Prior to initiation of these studies, approval was obtained for research on human materials (QEII Health Sciences Center, Research Ethics Committee, no. QE-RS-99-016). Table 2-1 indicates ovarian cancer number (OC#) of all patient samples used in this study. Approximately 2-4 litres of ascites fluid was obtained from patients at late stage ovarian cancer. Ascites fluid was mixed 1:1 with growth medium (MCDB105 (Sigma)/M199 (Invitrogen) supplemented with 10% fetal bovine serum (FBS; CanSera) and 1X penicillin/streptomycin) in T75 tissue culture flasks. Cells were allowed to adhere for 3-4 days, washed 2 times with phosphate buffered saline (PBS), and fed fresh growth medium. All experiments using primary human ovarian cancer cells were performed at passages 1-8. Alternative DMEM/F12 (Invitrogen) culture medium supplemented with 3% FBS, 5 μg/ml insulin, 0.05 mM ethanolamine, 5 ng/ml EGF, 10 μg/ml transferrin and 1X penicillin/streptomycin (DMEM/F12 mix) was tested to assess whether passage number could be enhanced.

2.2 Cell lines

Human ovarian cancer cell lines used for this study include SkOV3 and CaOV3 cells. SkOV3 and CaOV3 cells are derived from patients with ovarian adenocarcinoma. Ovarian cancer cell lines were maintained in DMEM (Invitrogen) supplemented with 5% FBS and 0.1 mM nonessential amino acids. Mink lung epithelial cells (Mv1Lu) are used as a positive control for TGFβ responsiveness. Mv1Lu cells were maintained in DMEM supplemented with 5% FBS.

2.3 Transient transfections and luciferase assays

Ovarian cancer cell lines were plated at a density of 4 x 10^4 cells/well in 12 well plates on day 0. On day 1, cells in triplicate wells were transfected with 300ng reporter plasmid. Positive control wells were transfected with pCMV-luc, and negative controls included pCMV, pGL2-modified and pGL2-basic. The TGFβ responsive reporter plasmid, p3TP-lux (Carcamo et al. 1995) was used to assess TGFβ responsiveness. Cells were transfected using FuGENE6 transfection reagent (Roche Molecular Biochemicals, Laval, Quebec) in normal growth medium. FuGENE6 transfection reagent (3 μl) was mixed with serum free medium (50 μl), and incubated for 5 min at room temperature. 300 ng of either pCMV, pGL2-basic, pGL2-mod, or p3TP-lux, in combination with 300 ng of pCMV-ßgal was added to the FuGENE6 mix, and incubated for 15 min at room temperature. Cells were fed fresh complete growth medium, and the FuGENE6:DNA mix was added to each well. On day 2, cells were serum starved (0.2 % FBS) for approximately 8h, and treated with TGFβ (Sigma-Aldrich Corp., Oakville, Ontario, Canada). An initial dose response was conducted using 0.1 ng/ml, 1 ng/ml and 10 ng/ml TGFß. Approximately 20h after treatment, cells were harvested for luciferase assay using the Enhanced luciferase assay kit (BD Pharmingen, Mississauga, Ontario). Cells were washed 3 times with PBS, and lysed using Luciferase lysis buffer (Roche), and assayed for luciferase activity using a Luminometer (Lumat LB 9507, Berthold Technologies). Transfection results were normalized by a ß-galactosidase (ßgal) assay. Cell lysate was added to buffer Z (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1mM $MgSO_4$), and o-Nitrophenyl ß-D-galactopyranoside (ONPG) substrate in 0.1M Phosphate buffer. The reaction was incubated at 28°C until formation of a yellow color,

at which time, the reaction was stopped with 1M Na_2CO_3. Absorbance of samples was read at 420 nm. Results shown are representative from at least 3 independent experiments.

2.4 Total RNA extraction

Total RNA for reverse transcription polymerase chain reaction (RT-PCR) was either isolated using the guanidinium isothiocyanate method (Chomczynski P and Sacchi N 1987), or isolated using the Sigma GenElute total RNA isolation kit (Sigma) followed by DNaseI treatment to remove potential genomic DNA contamination. RNA used for Northern blots was isolated using the Sigma GenElute total RNA kit. Primary ovarian cancer cells and cell lines grown to 70-80% confluence were treated with 0.1 ng/ml TGFß, 1 ng/ml TGFß, 10 ng/ml EGF alone or in combination for 4h, as indicated in figure legends, prior to harvesting for RNA isolation.

2.5 RT-PCR

cDNA synthesis was conducted using 3 μg of total RNA isolated from primary ovarian cancer cells and ovarian cancer cell lines. Total RNA (3 μg) was incubated with oligo $d(T_{12-18})$ (0.5 μg), 10mM dNTPs, 0.1M DTT, and reverse transcriptase (200 μg; Superscript II; Invitrogen) for 50 min at 42°C. PCR was then conducted using 1 μl of cDNA as template and the primer sets indicated in Table 5. PCR reactions were done for 30cycles: 35 sec at 94°C, 35 sec at 59.5°C, 35 sec at 72°C, with the exception of E-cadherin, which was done for 30 cycles: 60 sec at 94°C, 90 sec at 60°C, 120 sec at 72°C.

2.6 Northern analysis

Ten micrograms of total RNA was separated on 1.5% formaldehyde gels and transferred to Brightstar plus membrane (Ambion Inc., Austin, TX). Blots were pre-

incubated in hybridization buffer (see below) for 2-4h followed by incubation with 1 x 10^6 cpm/ml probe overnight in hybridization buffer. Signals were visualized by autoradiography and loading controlled by normalization to the GAPDH signal. Data shown is representative of at least 2 independent experiments.

2.6.1 cRNA probes: Isolated PCR products (see section 2.5) were subcloned into pCRII-TOPO , orientation determined by restriction enzyme analysis, followed by linearization with EcoRV or XbaI. ^{32}P-labeled antisense cRNA probes ($p15^{INK4B}$) were generated by incubation of the cDNA fragment with the SP6 RNA polymerase, 10mM NTP (ATP, CTP, GTP), transcription buffer (MBI), RNase inhibitor, and ^{32}P-UTP for 1h at 37°C. ^{32}P-labeled probes were purified by polyacrylamide gel electrophoresis and counted by scintillation. Blots were incubated with cRNA probes overnight at 60°C in hybridization buffer (400mM sodium phosphate, 1mM EDTA, 0.5% SDS, 1 mg/ml BSA, 50% formamide) and washed at 2 x 1h at 60°C and 1 x 15 min at 75°C in wash solution (0.1%SDS, 0.1X SSC, 1mM EDTA).

2.6.2 cDNA probes: Isolated PCR products (see Section 2.5) were subcloned into pCRII-TOPO, digested with EcoRI to isolate the fragment. Following purification of the fragments (Geneclean II; Bio101, Rutherford, CA), ^{32}P-labeled cDNA probes were generated using the StripEZ DNA probe synthesis kit (Ambion Inc., Austin, Tx), purified using Sephadex G50 spin columns (Roche) and counted by scintillation. Blots were incubated with cDNA probes (Smad7, PAI-1, GAPDH) overnight at 42°C in ULTRAhyb buffer (Ambion Inc., Austin, Tx) and washed 2 x 5 min at 42°C in low stringency wash solution (0.1% SDS, 2X SSC) and 2 x 15 min in high stringency wash solution (0.1%SDS, 0.1X SSC).

2.7 Protein extraction

Whole cell protein extracts or nuclear and cytoplasmic protein extracts were prepared from primary ovarian cancer cells and cell lines grown to about 70-90% confluence. Ovarian cancer cells were serum starved (growth medium supplemented with 0.2% FBS) overnight followed by treatment for Smad Western blots. Cells were treated with 0.1 ng/ml TGFß and/or 10 ng/ml EGF for 1h (for all Smad Westerns) or 4h ($p15^{INK4B}$ Westerns) prior to harvesting protein. Protein extracted for phospho-Smad2 and phospho-Smad3 Western blots were prepared by lysing cells in cell lysis buffer containing 50mM HEPES, 150mM NaCl, 10% glycerol, 1.5mM MgCl2, 1mM EGTA, 1mM sodium vanadate, 10mM sodium pyrophosphate, 10mM NaF, 1% triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1mM PMSF and 1X protease inhibitor cocktail (Roche Molecular Biochemicals, Laval, Quebec). Protein extracted for $p15^{INK4B}$ Western blots were prepared by lysing cells in cell lysis buffer containing 50mM Tris-HCl (pH 7.6), 150mM NaCl, 1mM EDTA, 0.5% NP40, 0.1% triton X-100 and 1X protease inhibitor cocktail. For some experiments, nuclear and cytoplasmic protein was isolated separately. Cytoplasmic protein was isolated by lysing the cytoplasmic membrane with lysis buffer containing 10mM HEPES, 1.5mM $MgCl_2$, 10mM KCl, 1X protease inhibitor cocktail and 1mM PMSF. The cytoplasmic protein was separated from the nuclei by centrifugation (1310 xg for 10min at 4°C), and nuclear protein was isolated by lysing the nuclei in lysis buffer containing 20mM HEPES, 25% glycerol, 0.42M NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 1X protease inhibitor cocktail, and 1mM PMSF. Concentrations of all protein extracts were quantified using a Bradford assay, with BSA as a standard.

2.8 Western analysis

2.8.1 Smads: Twenty micrograms of whole cell protein or 10μg of nuclear protein from primary ovarian cancer cells and cell lines were separated on 12% polyacrylamide gels. Protein was transferred to nitrocellulose membranes, and blocked for 1h in 5% milk in TBS-T. Protein was detected using specific rabbit anti-human antibodies against Smad2 (1:1000; Zymed Laboratories Inc, South San Fransisco, CA), phospho-Smad2 (1:1000; Upstate biotechnology Inc, Lake Placid, NY), Smad3 (1:500; Zymed Laboratories Inc), phospho-Smad3 (1:2000; gift from Edward Leof, Mayo Clinic Cancer Center) and Actin (1:1000; Sigma, St. Louis, MO). Following incubation with peroxidase (POD)-conjugated anti-rabbit antibody (1:5000; Boerhinger Ingelheim), signals were detected using enhanced chemiluminescence (ECL; Perkin-Elmer corp., Markham, ON). Blots were stripped (62.5mM Tris pH 6.8, 2% SDS, 0.1M ß-mercaptoethanol) for 15 min at 55°C, blocked, and re-probed. Blots were initially probed with Smad2 or Smad3 antibodies, followed by re-probing with the phospho-specific antibodies, and lastly probed with anti-actin. Data is representative of at least 2 independent experiments.

2.8.2 $p15^{INK4B}$: Ten micrograms of whole cell protein was separated on 16% polyacrylamide gels and transferred to nitrocellulose membranes. Following blocking (1h at room temperature in 5% milk/TBS-T), membranes were incubated with rabbit polyclonal antibody specific for $p15^{INK4B}$ (1:200; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Following incubation with POD-conjugated anti-rabbit antibody (1:5000; Boerhinger Ingelheim), signals were detected using ECL. Data is representative of at least 2 independent experiments.

2.9 Immunofluorescence

Primary ovarian cancer cells (OC5, OC15, OC24, OC32) were plated on glass coverslips in 12 well plates at a density of 4 x 10^4 cells/ well and allowed to adhere for 24h. Cells were then serum starved overnight and the following day, cells were treated with TGFß (1 ng/ml) and EGF (10 ng/ml) for 1h. Cells were fixed with 4% paraformaldehyde, blocked in 5% horse serum, and incubated with primary antibody overnight at 4°C. Rabbit anti-Smad2 (2.5 μg/ml) and anti-Smad3 (10 μg/ml) antibodies were used to detect cellular localization of Smad2 and Smad3 protein. Fluorescent conjugated anti-rabbit IgG Alexa Fluor 488 (Molecular Probes, Eugene OR) was used to detect protein expression and intracellular localization by immunofluorescence. Data is representative of 2 independent experiments.

2.10 Growth curves

Primary ovarian cancer cells and ovarian cancer cell lines were plated at a density of 4 x 10^4 cells/well in a 6 well plate on day 0. On day 1 and every third day, cells were treated with the various ligands in normal growth media. Treatments included serum starvation (0.2% FBS), control (10% FBS), TGFβ (0.1 ng/ml or 1 ng/ml), and EGF (1 ng/ml, 5 ng/ml or 10 ng/ml), alone and in combination. Cells were counted over a 6-9 day period using a hemacytometer. Data is representative of at least 3 independent experiments.

2.11 Trypan blue exclusion

Trypan blue exclusion was used to determine if cell viability was affected by the various treatments. Primary ovarian cancer cells (OC3, OC5, OC7) were plated at 2 x 10^4 cells/well in 12 well plates, and treated with 0.1 ng/ml TGFß the following day. On

d4, cells were lifted and mixed 1:1 with 0.4% trypan blue and counted using a hemacytometer. Viable cells do not retain the trypan blue and remain clear, whereas non-viable cells have altered membrane integrity and will stain blue. Percent viability was assessed by determination of the number unstained cells versus total number of cells.

2.12 ELISA

TGFβ was measured in ascites fluid obtained from ovarian cancer patients using Quantikine human TGFß1 Immunoassay (R&D Systems, Minneapolis, MN). Ascites samples were either activated to detect both latent and active TGFß1, or left untreated to detect only active TGFß1. Activation was done by acidification, followed by neutralization according to manufacturer specification. Samples and standards (0 – 2000 pg/ml recombinant TGFß) were added to wells of a 96-well plate precoated with type II TGFß receptor (TßRII). Wells were washed, and horse radish peroxidase (HRP)-conjugated polyclonal antibody against TGFß1 was added. Following incubation, wells were washed, and substrate solution was added. After stop solution was added, the optical density was determined at 450nm, and TGFß1 concentrations were determined using a standard curve.

EGF was measured in ascites fluid obtained from ovarian cancer patients using the Quantikine human EGF Immunoassay (R&D Systems). Standards (0 – 250 pg/ml) and samples (1:20 dilution, 200 μl) were added to wells of a 96-well plate precoated with anti-EGF antibody. Wells were washed, and HRP-conjugated polyclonal antibody specific for EGF was added. Following incubation, wells were washed, and substrate solution was added. After the reaction was stopped by the addition of the stop solution,

the optical density was measured at 450nm and EGF concentrations were determined using a standard curve.

2.13 [^{3}H]-thymidine incorporation assay

Mv1Lu cells were plated at a density of 3 x 10^4 cells/well in 24 well plates on d0. On d2, cells were treated with ascites fluid (0.1ng/ml and 0.5ng/ml ascitic TGFß) or recombinant TGFß (0 – 2.5 ng/ml) and [^{3}H]thymidine (1 μCi/well) in serum free medium for 36-48h at 37°C. Cells were rinsed with PBS, and incubated with 10% trichloracetic acid (TCA) to precipitate DNA. TCA was removed, cells were rinsed with absolute ethanol and allowed to dry. 0.1M NaOH was used to dissolve the monolayer, and incorporated [^{3}H]thymidine was measured by scintillation.

2.14 TGFß neutralization

TGFß activity was neutralized in some experiments to confirm that activity was dependent on TGFß present in ascites fluid, and not due to other factors. Antibody specific for TGFß (5 μg/ml – 20 μg/ml) (R&D systems) was pre-incubated with ascites fluid or TGFß for 30 min at 37°C. Following incubation, the antibody and ascites was added to ovarian cancer cells or Mv1Lu cells, and activity assessed by [^{3}H]-thymidine incorporation or Western analysis.

2.15 Statistical analysis

Statistical analysis of growth curves was done using a repeated measures two-way ANOVA with Tukey's post hoc test ($P < 0.05$). Statistical analysis of the transfection data was done using an unpaired t-test ($P < 0.05$).

OC #	Histology	Stage
1	serous	3B
2	serous	
3	clear cell adenocarcinoma	
4	serous, poorly differentiated	3C
5	mucinous	
6	poorly differentiated	
7	serous	
8	mixed	3C
9	serous	3C
10	undifferentiated	3C
11	serous	
12	serous	
13	NOT ovarian	
14	clear cell	
15	serous	
16	? No primary tumor	
17	mucinous	2A
18	serous	
19	poorly differentiated/serous	
20	poorly differentiated	
21	serous	
22	serous	
23	poorly differentiated	
24	serous	
25	serous	
26	poorly differentiated	
27	mucinous	
28		3C
29	serous	
30		
31	clear cell	3C
32	serous	
33	serous	3C
34	mucinous	1A
35	serous	
36	serous	3B
37	serous	3C
38	serous	3C
39	gastric - non ovarian	
40	serous	3C
41		
42	undifferentiated	3C
43	serous	3C
44		4
45		

Table 2-1: OC number, histology and stage of ovarian cancer samples.

Product	5' primer	3' primer	Product
E-cadherin	gggtgactacaaaatcaatc	gggggcagtaagggctcttt	250
TGFß	cgccttagcgcccac	aggcgcccgggttat	400
TßRI	gagcatggatccctttttga	aacatcgtcgagcaatttcc	397
TßRII	ataaggccaagctgaagcag	gagctcttgaggtccctgtg	373
Smad2	cgaaatgccacggtagaaat	ctgccttcggtattctgctc	327
Smad3	atattccagagaccccaccc	ctccgatgtagtagagccgc	378
Smad4	ctgccaactttcccaacatt	gatctcctccagaagggtcc	450
Smad7	caacttcttctggagcctgg	caaagctgatctgcacggta	451
SARA	gtgtgttggattggcagatg	gcaccaccaaaaggaacact	423
$p15^{INK4B}$	ggactagtggagaaggtgcg	aaataaagtcgttgtgggcg	381
$p21^{CIP1}$	gacaccactggagggtgact	ctcttggagaagatcagccg	318
PAI-1	caacgtggttttctcaccct	gggcgtggtgaactcagtat	559
TGIF	tggcagtgagactgaggatg	gcgggaaattgtgaactgat	304
GAPDH	tggaaatcccatcaccatct	gtcttctgggtggcagtgat	351

Table 2-2: Primers and product sizes for RT-PCR

CHAPTER 3

PRIMARY HUMAN OVARIAN CANCER CELLS MAINTAIN AN INTACT TGFß SIGNALING PATHWAY

3.1 SUMMARY

Defects in the TGFß signaling pathway occur in numerous types of cancer. Mutations in components of the TGFß signaling pathway have been described in cancer, resulting in resistance to the anti-proliferative effect of TGFß. TGFß signals through a receptor complex, consisting of type I and type II subunits, which upon activation by ligand binding, activate intracellular signaling molecules known as Smads. TGFß signaling defects have been described in ovarian cancer cells, however, the functional significance of these defects is unknown. Furthermore, studies examining the anti-proliferative effect of TGFß in ovarian cancer cells have been contradictory. The hypothesis of this study is that TGFß signaling defects occur in ovarian cancer cells, resulting in resistance to the anti-proliferative effect of TGFß. Expression of TGFß type I and type II receptors as well as the Smads responsible for TGFß signaling was assessed, and all components are expressed. Furthermore, the components are functional, considering TGFß is able to up-regulate endogenous and transfected target genes in primary ovarian cancer cells and cell lines. In addition, primary ovarian cancer cells respond to the anti-proliferative effect of TGFß, which is likely mediated by TGFß induced up-regulation of $p15^{INK4B}$ expression. In contrast, ovarian cancer cell lines are resistant to the anti-proliferative effect of TGFß, which is likely due to a $p15^{INK4B}$ gene deletion. Therefore, disruptions in the TGFβ signaling pathway appear to be an uncommon event in ovarian cancer, however, mechanisms must exist to modulate TGFß signaling since ovarian cancer cells continue to proliferate *in vivo*.

3.2 INTRODUCTION

TGFß inhibits proliferation of normal epithelial cells, including the OSE. Resistance to the anti-proliferative effect of TGFß has been reported in a number of different cancers. Colorectal, lung, and pancreatic cancers commonly have defects in TGFß signaling components, contributing to uncontrolled cellular proliferation of cancer cells. Mutations in TßRI (Kim et al. 1996; DeCoteau et al. 1997; Chen et al. 1998; Chen et al. 1999; Kang et al. 1999), TßRII (Park et al. 1994; Markowitz et al. 1995; Myeroff et al. 1995; Osada et al. 2001), Smad2 (Eppert et al. 1996; Uchida et al. 1996; Riggins et al. 1997; Prunier et al. 1999) and Smad4 (Hahn et al. 1996; Nagatake et al. 1996; Takagi et al. 1996) have been detected in cancer, causing signaling defects.

Receptor and Smad mutations have been described in ovarian cancer, however, it is unclear whether these mutations result in signaling defects (Chen et al. 2001; Francis-Thickpenny et al. 2001; Lynch et al. 1998; Wang et al. 2000). Furthermore, studies examining the anti-proliferative effect of TGFß on ovarian cancer cells have been contradictory. Both resistance and sensitivity to the anti-proliferative effect of TGFß in ovarian cancer cells have been reported. Berchuck et al. (1992) showed that established ovarian cancer cell lines are resistant to the anti-proliferative effect of TGFß (Berchuck et al. 1992). Research conducted using primary ovarian cancer cells have shown both resistance to the anti-proliferative effect of TGFß (Yamada et al. 1999), as well as sensitivity to the anti-proliferative effect of TGFß (Hurteau et al. 1994).

Since TGFß signaling defects are implicated in other types of cancer, this study hypothesized that TGFß signaling defects may contribute to ovarian tumorigenesis. Moreover, due to the contradictory results in the literature, further examination of the

TGFß signaling pathway is necessary to determine if primary ovarian cancer cells and ovarian cancer cell lines respond to the anti-proliferative effect of TGFß. The TGFß signaling pathway was found to be intact in primary ovarian cancer cells and ovarian cancer cell lines. Primary ovarian cancer cells were found to respond to the anti-proliferative effect of TGFß, which is in part mediated by an increase in $p15^{INK4B}$ mRNA expression in response to TGFß. Conversely, ovarian cancer cell lines and OC1 do not respond to the anti-proliferative effect of TGFß, and this is likely due to the $p15^{INK4B}$ gene deletions that occur in the ovarian cancer cell lines, as well as OC1.

3.3 MATERIALS AND METHODS

All methods were done according to those described in Chapter 2. Primary ovarian cancer cells used for growth curves, protein and RNA extraction were grown in complete MCDB105/M199 medium. Only for the purpose of characterization were primary ovarian cancer cells grown in the DMEM/F12 mix.

3.4 RESULTS

3.4.1 Primary ovarian cancer cell characterization

The typical cobblestone morphology of primary ovarian cancer cells grown in monolayer culture is shown in Figure 3-1A, however, a larger cellular phenotype can also occur (Figure 3-1B). Occasionally, stromal contamination occurs (Figure 3-1C) and these cells will eventually over grow any epithelial cells present. Primary ovarian cancer cells grown in the MCDB105/M199 culture medium consistently displayed a phenotype

of epithelial cells, whereas cells grown in the DMEM/F12 mix underwent a phenotypic change and exhibited a spindle shaped cell type.

E-cadherin mRNA expression was analyzed to distinguish primary ovarian cancer cells from potential mesothelial contaminants, which do not express this marker (Schofield et al. 1997; Kitazume et al. 2000). E-cadherin mRNA was expressed in primary ovarian cancer cell samples OC3-7, OC11, OC12, OC14, OC25 (Figure 3-2 and data not shown).

3.4.2 Histology and stage of primary ovarian cancers

Table 2-1 indicates the OC numbers, histology and stage of ovarian cancer. The age range of the patients is 36-86, however the majority of patients are 60-80. Serous adenocarcinoma is the most common histology, and although stage was not available for all patients, the majority are at late stages (3B, 3C).

3.4.3 All known components of the TGFβ signaling pathway are expressed in primary ovarian cancer cells and cell lines

To determine whether all known components of the TGFß signaling pathway are expressed in primary ovarian cancer cells and cell lines, RT-PCR analysis was done using RNA extracted from these cells. TGFß1, TßRI, TßRII, Smad2, Smad3, Smad4 and SARA are expressed in primary ovarian cancer cells and ovarian cancer cell lines (Figure 3-3). Results are shown for OC1, and identical results were found for all primary ovarian cancer cells and cell lines tested (OC1-12, OC31, OC33-37).

3.4.4 The TGFß signaling pathway is functional in ovarian cancer cell lines

In order to determine whether the components of the TGFß signaling pathway were functional in ovarian cancer cell lines (CaOV3 and SkOV3) and OC1, transfections

using a TGFß responsive reporter gene, p3TP-lux were done. Ovarian cancer cell lines and OC1 were transfected with p3TP-lux, treated with 0.1 ng/ml TGFß, and luciferase activity was assessed. Following transfection and treatment with TGFß, a significant increase in luciferase activity was seen in SkOV3 cells (Figure 3-4A) and results were similar for CaOV3 and OC1 (data not shown). Dose response using 0.1 ng/ml to 10 ng/ml TGFß (Figure 3-4B) shows the response seen with 0.1 ng/ml TGFß is similar to that seen with 10ng/ml TGFß, therefore 0.1 ng/ml TGFß was used for all future experiments.

3.4.5 The TGFß signaling pathway is functional in primary ovarian cancer cells

We determined that primary ovarian cancer cells are unable to be efficiently and consistently transfected following attempts with several transfection methods. FuGENE6, calcium phosphate, lipofectamine, and Effectene were all tested, and either resulted in cell death (Effectene) or inconsistent transfection efficiency. Therefore, endogenous TGFß target gene expression was used to assess the TGFß pathway. Northern analysis of the TGFß target genes PAI-1 and Smad7 indicates that TGFß increases expression of both target genes in primary ovarian cancer cells (Figure 3-5). PAI-1 and Smad7 mRNA expression is minimal in untreated primary ovarian cancer cells, and is increased by TGFß treatment.

3.4.6 Primary ovarian cancer cells respond to the anti-proliferative effects of TGFß

Growth curves were conducted on the primary ovarian cancer cell cultures to determine whether they responded to the anti-proliferative effects of TGFß. Primary ovarian cancer cells grown under normal growth conditions (growth medium supplemented with 10% FBS) have a normal rate of proliferation, and serum starved cells

(normal growth medium with 0.2% FBS) stop growing. Proliferation is inhibited in primary ovarian cancer cells treated with 0.1ng/ml TGFß (Figure 3-6). Results are shown for OC2, OC3, OC5, and OC7, and similar results were obtained for OC4 and OC6.

3.4.7 Cell viability is not affected by TGFß

Trypan blue exclusion was used to assess viability of primary ovarian cancer cells. Viable cells do not retain trypan blue and remain clear, whereas non-viable cells have altered membrane integrity and retain the blue color. Viability was similar in untreated and TGFß (0.1ng/ml) treated primary ovarian cancer cells OC3, OC5 and OC7 (Figure 3-7).

3.4.8 Primary ovarian cancer cells up-regulate $p15^{INK4B}$ mRNA and protein expression in response to TGFß

The anti-proliferative effect of TGFß is partially mediated by up-regulation of the cell cycle regulator $p15^{INK4B}$. $p15^{INK4B}$ mRNA expression is limited in untreated cells (-), and TGFß treatment (+) strongly enhances $p15^{INK4B}$ mRNA expression in OC2, OC8-10, and a normal OSE sample, NOSE1 (Figure 3-8A). In contrast SkOV3 and OC1 cells do not appear to express $p15^{INK4B}$ mRNA. OC2 and OC3 cells up-regulate $p15^{INK4B}$ protein expression in response to TGFß treatment, whereas SkOV3 and OC1 cells do not express $p15^{INK4B}$ protein (Figure 3-8B).

3.4.9 Ovarian cancer cell lines and OC1 are resistant to the anti-proliferative effect of TGFß

Since ovarian cancer cell lines and OC1 have deleted the gene for $p15^{INK4B}$ (Watson et al. 1999; Dunfield et al. 2002), the anti-proliferative effect of TGFß was tested in these cells to determine if $p15^{INK4B}$ deletions affected the TGFß response.

Ovarian cancer cell lines and OC1 have a normal rate of proliferation when grown under normal growth conditions, as well as when grown in the presence of 0.1 ng/ml TGFß (Figure 3-9A). Increasing doses (0.1 ng/ml – 10 ng/ml) of TGFß does not alter this response (Figure 3-9B).

3.5 DISCUSSION

This study characterizes primary ovarian cancer cells isolated from ascites fluid of late stage ovarian cancer patients and grown in monolayer culture and investigates the TGFß signaling pathway in these cells. Primary ovarian cancer cells have an intact and functional TGFß signaling pathway, and they respond to the anti-proliferative effect of TGFß, partially by TGFß-induced up-regulation of $p15^{INK4B}$ expression.

Primary ovarian cancer cells isolated from ascites fluid exhibit the typical cobblestone morphology of ovarian cells grown in monolayer culture (Auersperg et al. 1994; Figure 3-1). In our experience, the cobblestone epithelial cells grow well and are most useful for experimentation. Ovarian cancer cells have a limited growth potential (6-8 passages) and will eventually exhibit the phenotype of senescent cells that fail to divide. Therefore the optimal time to conduct most experiments is when the cells are at a low passage number. An alternative growth medium for primary ovarian cancer cells was tested to determine whether passage number could be increased. Evangelou et al. (2000) grow primary ovarian cancer cells in DMEM/F12 supplemented with 3% FBS, ethanolamine, insulin, EGF, transferrin and penicillin/streptomycin (Evangelou et al. 2000). We found, however, that the morphology of primary ovarian cancer cells is altered when grown under these conditions. These cells are spindle shaped, and no

longer have an epithelial phenotype. This altered phenotype is likely due to the EGF present in this medium, which has been shown to alter phenotype and cell characteristics (Siemens and Auersperg 1988). Furthermore, cell passage number was not extended under these conditions and therefore this type of medium was not used for further experiments. Histology and stage of the ovarian cancer (Table 2-1) is not correlated with the various phenotypes.

CA-125 is a high molecular weight serum glycoprotein complex present in >80% of epithelial ovarian cancer patients (Hempling 1994). CA-125 is a common marker used for ovarian cancer detection and prognosis, however, results using this marker are inconsistent and unreliable (Kabawat et al. 1983; Hempling 1994; Tortolero-Luna et al. 1994). Hence, usefulness of CA-125 to confirm identity of ovarian cancer cells is limited. Normal OSE is a simple mesothelium, which differentiates to a more epithelial phenotype during neoplastic progression (Auesperg et al. 1998). E-cadherin is an epithelial marker which is never expressed in mesothelial cells and can be used to distinguish reactive mesothelium from cancer cells (Scholfield et al. 1997). E-cadherin is expressed in ovarian epithelial cancer and normal OSE from patients at high risk for ovarian cancer (Maines-Bandiera and Auersperg 1997; Sundfeldt et al. 1997; Wong et al. 1999). All primary ovarian cancer cells tested express E-cadherin mRNA (Figure 3-2). Potential mesothelial contaminants would not express E-cadherin (Scholfield et al. 1997), and demonstrate a smaller morphology compared to the ovarian cancer cells (Dr.S. Murray, Dept. of Pathology, QEII Health Science Centre, personal communication). In addition, contaminating stromal cells will quickly outgrow the ovarian epithelial cancer cells (Figure 3-1C), and these samples are discarded. Therefore, based on E-cadherin

expression and morphology we conclude that the cells isolated from ascites fluid are indeed primary ovarian cancer cells and not reactive mesothelial cells or stromal contaminants.

This work shows that expression of all components of the TGFß signaling pathway are expressed in ovarian cancer cell lines and primary ovarian cancer cells as analyzed by RT-PCR (Figure 3-3). TGFß1, TßRI, TßRII, Smad2, Smad3, Smad4 and SARA are expressed in all primary ovarian cancer cell samples tested (OC1-12, OC31, OC33-37) and ovarian cancer cell lines (SkOV3, CaOV3). As all components of the TGFß signaling pathway were expressed, we next determined whether these components were functional and could transmit a TGFß signal. The TGFß signaling pathway was found to be intact in ovarian cancer cell lines, as measured by luciferase activity of a TGFß-responsive reporter gene, p3TP-lux (Figure 3-4). SkOV3, CaOV3, and OC1 cells all up-regulate p3TP-lux activity in response to TGFß. TGFß signaling was measured in primary ovarian cancer cells by examination of the endogenous target genes Smad7 and PAI-1 (Figure 3-5). TGFß treatment up-regulates Smad7 and PAI-1 mRNA in all ovarian cancer cells tested, indicating that the TGFß signaling pathway is intact in primary ovarian cancer cells.

To determine whether primary ovarian cancer cells would respond to a cellular effect of TGFß, growth curves were done to assess the proliferative response of primary ovarian cancer cells to TGFß. TGFß induces growth arrest in normal OSE (Berchuck et al. 1992). Primary ovarian cancer cells were found to respond to the anti-proliferative effect of TGFß (Figure 3-6). In agreement with this study, Hurteau et al. (1994) also demonstrated that primary ovarian cancer cells are sensitive to the anti-proliferative

effects of TGFß. In contrast, Yamada et al. (1995) showed that primary ovarian cancer cells are resistant to the anti-proliferative effect of TGFß. The study by Hurteau et al. used primary ovarian cancer cells isolated from ascites fluid, and examined changes in proliferation using a [^{3}H]-thymidine incorporation assay. Yamada et al. (1999) studied proliferation of primary ovarian cancer cells isolated from tumor extracts, and used an MTT assay to examine changes in proliferation. The origin of the primary ovarian cancer cells (ascites vs. solid tumor explants) may account for these contradictory results. In addition, Yamada et al. studied proliferation using a MTT assay over a 48h period, whereas the present studied used cell counts over a 6-9d period. Indeed, little effect on proliferation was seen at the shorter time points, and significant differences in proliferation were only seen after 4-5d (Figure 3-6). Therefore, it is likely that the time course of the study by Yamada et al. was not sufficient to see significant changes in proliferation.

TGFß can induce apoptosis in normal and cancer cells (Havrilesky et al. 1995; Choi et al. 2001). Therefore, to ensure the results seen in Figure 3-6 were due to growth arrest, and not an induction of apoptosis, cell viability was assessed. Although apoptosis was not measured directly, trypan blue exclusion was used to assess viability of primary ovarian cancer cells treated with TGFß. Viability of primary ovarian cancer cells was not affected by TGFß treatment, indicating that TGFß does not induce apoptosis in these cells (Figure 3-7) and the growth inhibitory response seen in Figure 3-6 is due to cell cycle arrest.

The anti-proliferative effect of TGFß is partially mediated by up-regulation of the cell cycle regulator, $p15^{INK4B}$ (Hannon and Beach 1994). Northern blots examining

$p15^{INK4B}$ mRNA expression in the primary ovarian cancer cells indicates that TGFß induces $p15^{INK4B}$ mRNA expression in these cells, which likely contributes to the anti-proliferative effect of TGFß. In contrast to the primary ovarian cancer cells, ovarian cancer cell lines and OC1 do not express $p15^{INK4B}$ mRNA (Figure 3-8A). SkOV3 cells are known to have deleted the gene for $p15^{INK4B}$ (Watson et al. 1999), therefore CaOV3 cells as well as OC1 were tested and found also to have deleted the gene for $p15^{INK4B}$ (Dunfield et al. 2002). $p15^{INK4B}$ protein expression was also assessed and OC2 and OC3 up-regulate $p15^{INK4B}$ protein in response to TGFß treatment, whereas SkOV3 and OC1 cells do not express $p15^{INK4B}$ protein (Figure 3-8B). Although OC1 cells are primary ovarian cancer cells, they appear to have spontaneously immortalized in culture, and exhibit characteristics of an ovarian cancer cell line. Initially OC1 had a typical phenotype of the primary ovarian cancer cells, and ovarian tissue from the same patient has the $p15^{INK4B}$ gene, therefore, deletion of the gene for $p15^{INK4B}$ *in vitro* may be the cause of the immortalization of OC1.

Since the ovarian cancer cell lines and OC1 do not express $p15^{INK4B}$, the anti-proliferative effect of TGFß was examined in these cells. SkOV3 and OC1 cells are resistant to the anti-proliferative effect of TGFß (Figure 3-9A). Previous studies have shown that SkOV3 cells and other ovarian cancer cell lines are resistant to the anti-proliferative effect of TGFß (Zeinoun et al. 1999; Berchuck et al. 1992), therefore, our data for the ovarian cancer cell lines is in agreement with the literature. Furthermore, increasing doses of TGFß had no effect on proliferation in SkOV3 cells (Figure 3-9B).

This study demonstrates the importance of using primary ovarian cancer cells as opposed to ovarian cancer cell lines for studies investigating proliferative responses.

Ovarian cancer cells lines have deleted genes necessary for proper growth regulation, and hence, are a poor model to investigate the TGFß signaling pathway. Moreover, OC1 has also deleted the $p15^{INK4B}$ gene, and this primary ovarian cancer cell sample appears to have spontaneously immortalized in culture, likely due to this deletion. A previous study shows that $p15^{INK4B}$ was not deleted in tumor tissue isolated from this patient (Dunfield et al. 2002) and therefore, is likely not a factor in the development of ovarian cancer. Interestingly, OC1 now behaves like SkOV3 and CaOV3 cells, in that it it resistant to the anti-proliferative effect of TGFß, and like the cell lines, is able to be transfected in contrast to all other primary ovarian cancer cell samples. Due to the immortalization, this primary ovarian cancer cell sample was not used in further studies.

Although defects in the TGFß signaling pathway have been implicated in other types of cancers, the TGFß signaling pathway remains intact and functional in primary ovarian cancer cells. The anti-proliferative effect of TGFß is partially mediated by the up-regulation of $p15^{INK4B}$, and ovarian cancer cell lines that are resistant to the anti-proliferative effect of TGFß have deleted the gene for $p15^{INK4B}$ indicating this deletion to be a cause for TGFß resistance. This study demonstrates that primary ovarian cancer cells grown in monolayer culture are sensitive to the growth inhibitory effect of TGFß, suggesting TGFß may not play a role in ovarian tumorigenesis. This may not be the case, however, since TGFß is present *in vivo* and ovarian cancer cells continue to proliferate. In addition, TGFß can promote tumorigenesis and perhaps factors produced *in vivo* alter the TGFß response.

Figure 3-1. Primary ovarian cancer cell morphology. A) Typical cultures yield a cobblestone epithelial morphology (400X). B) Another common morphology is the larger, flattened epithelial cells (400X). C) Stromal contamination **(s)** can also occur, and these cells will eventually overtake the epithelial cells **(e)** (100X).

Figure 3-2: E-cadherin mRNA expression. RT-PCR analysis shows expression of E-cadherin mRNA in primary ovarian cancer cells OC5, 3, 14, 25, 7, 12. No E-cadherin mRNA expression is present in the no cDNA control (neg).

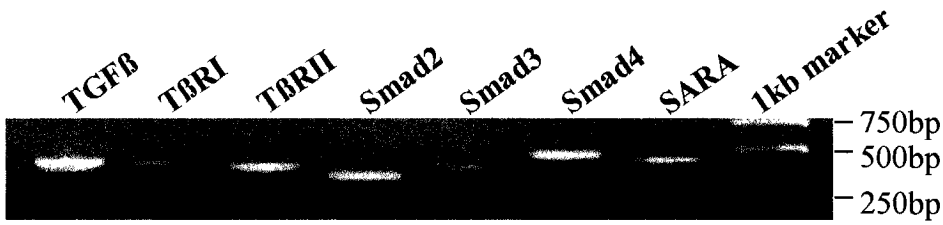


Figure 3-3: Expression of components of the TGFß signaling pathway. RT-PCR analysis shows expression of TGFß1, TßRI, TßRII, Smad2, Smad3, Smad4 and SARA in primary ovarian cancer cells and ovarian cancer cell lines. Representative data from OC1.

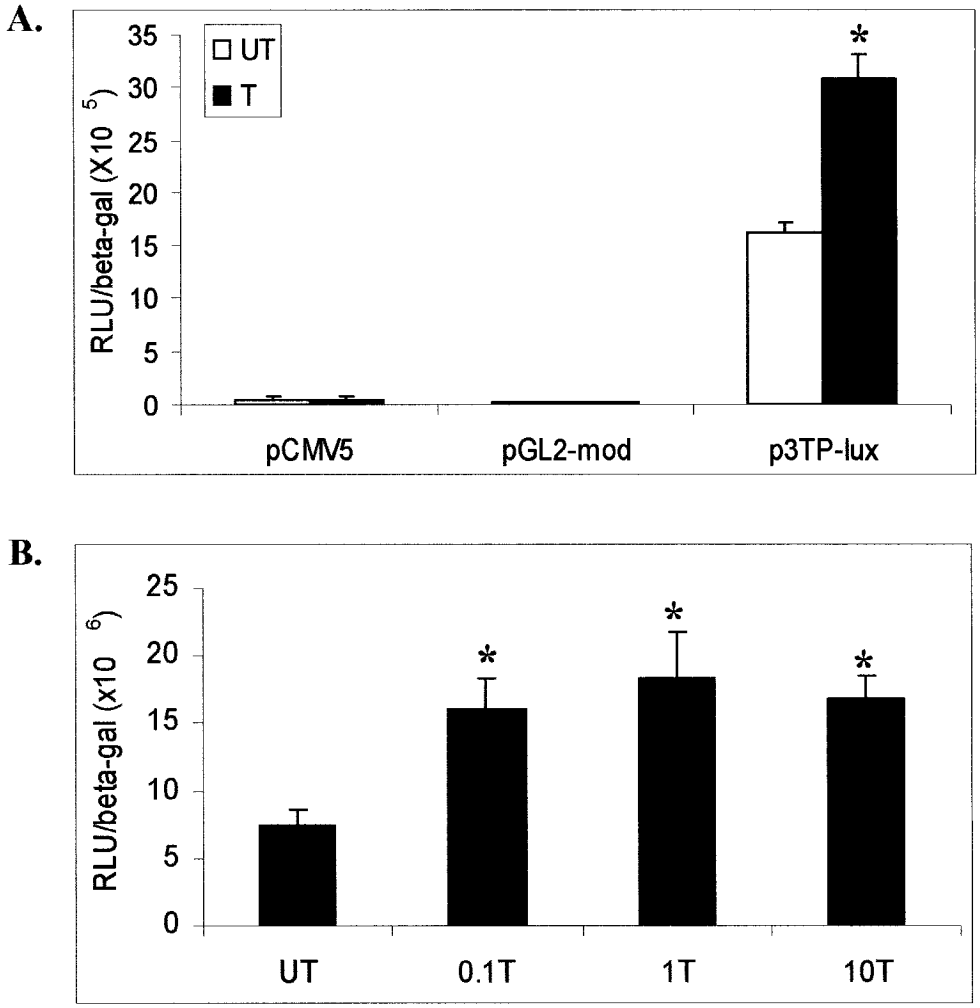
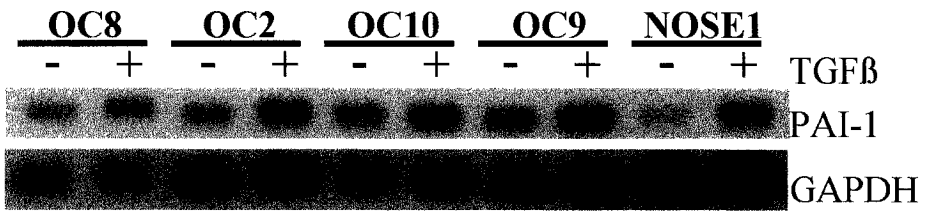


Figure 3-4: Ovarian cancer cell lines have an intact TGFß signaling pathway. A) SkOV3 cells transfected with pCMV5 have little luciferase activity (0.43 x10^5 RLU), which is not affected by treatment with 0.1ng/ml TGFß (T). Transfection with a promoterless luciferase construct (pGL2-mod) results in little luciferase activity, which is unchanged with treatment. Untreated SkOV3 (UT) cells transfected with p3TP-lux have some luciferase activity, which is significantly increased by treatment with TGFß (T). **B)** A dose response using 0.1ng/ml (0.1T), 1ng/ml (1T) and 10ng/ml (10T) TGFß results in a similar increase in luciferase activity with all concentrations of TGFß. Bars represent standard error of the mean (SEM). Statistical analysis was done using a t test (*, $P < 0.05$)

A.



B.

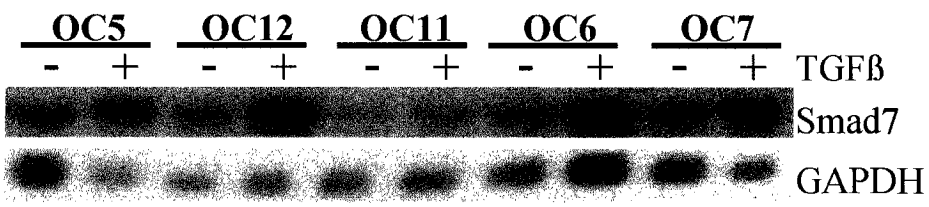


Figure 3-5: Primary ovarian cancer cells have an intact TGFß signaling pathway. Untreated (-) primary ovarian cancer cells have PAI-1 mRNA expression, which is increased with TGFß (+) treatment (**A**). Smad7 mRNA expression is also increased by TGFß treatment in primary ovarian cancer cells (**B**). GAPDH mRNA expression is shown for loading control.

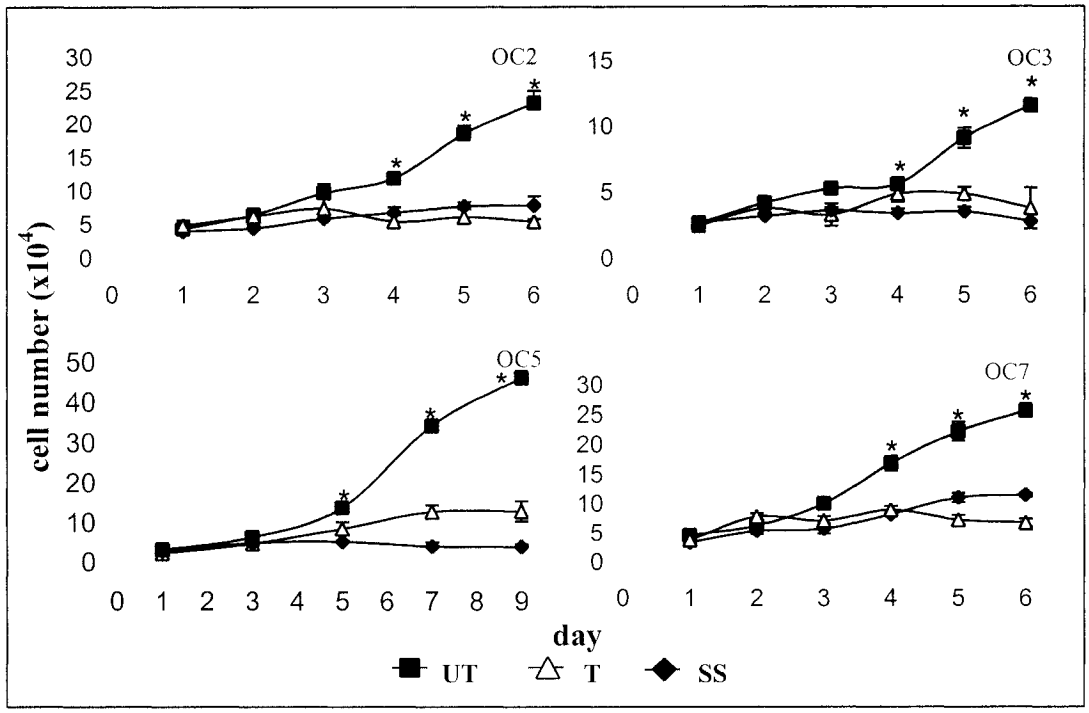


Figure 3-6: Primary ovarian cancer cells respond to the anti-proliferative effect of TGFß. Untreated (UT) primary ovarian cancer cells have a normal rate of proliferation, which is decreased in cells that are serum starved (SS; 0.2%FBS). Proliferation of primary ovarian cancer cells treated with 0.1ng/ml TGFß (T) is inhibited. The graphs show representative data from three separate experiments. Bars represent SEM. (* = significant difference between untreated and TGFß treated cells; $P<0.05$)

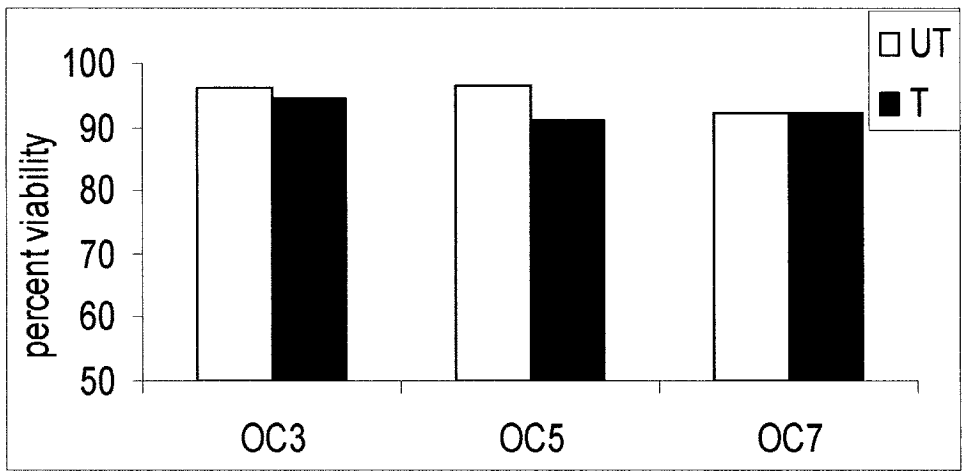


Figure 3-7: Viability of primary ovarian cancer cells is not affected by TGFß. Untreated primary ovarian cancer cells (UT) are over 90% viable, as measured by trypan blue exclusion. TGFß treatment (T) does not affect viability.

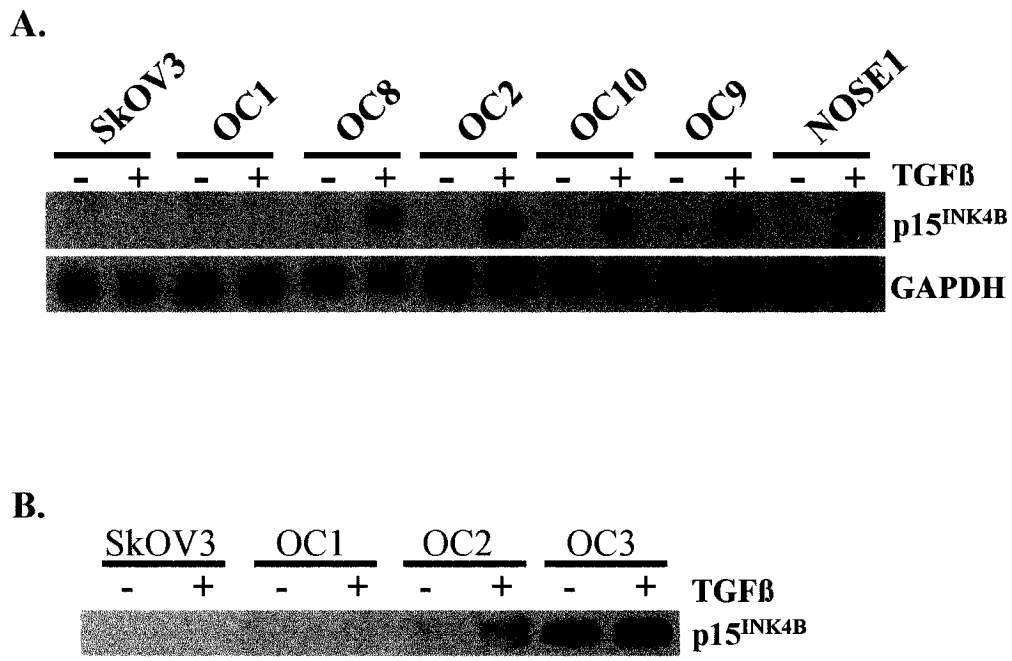


Figure 3-8: $p15^{INK4B}$ expression is increased in response to TGFß in primary ovarian cancer cells, and is not expressed in SkOV3 cells and OC1. A) Untreated cells (-) have no $p15^{INK4B}$ mRNA expression, and TGFß treatment (+) strongly induces $p15^{INK4B}$ mRNA expression in the primary ovarian cancer cells. $p15^{INK4B}$ mRNA is not expressed in untreated or TGFß treated SkOV3 cells or OC1. **B)** $p15^{INK4B}$ protein is not expressed in SkOV3 or OC1 cells. $p15^{INK4B}$ protein expression is increased in response to TGFß treatement in OC2 and OC3.

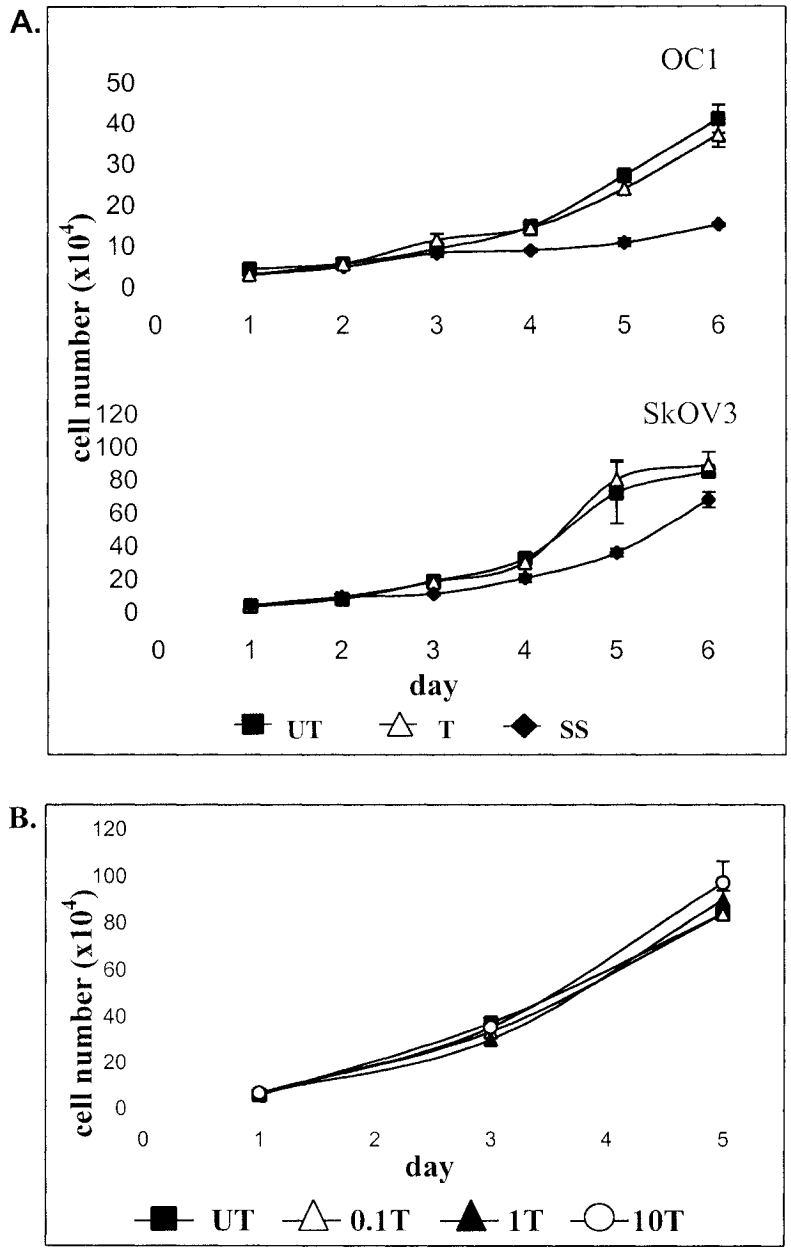


Figure 3-9. OC1 and SkOV3 cells are resistant to the anti-proliferative effect of TGFß. A. OC1 and SkOV3 cells have the same rate of proliferation when left untreated (UT) or treated with TGFß (T). Serum starved (SS) cells have a slower rate of proliferation. **B.** Increasing doses of TGFß does not affect proliferation in SkOV3 cells. Cells left untreated, treated with 0.1ng/ml TGFß (0.1T), 1ng/ml TGFß (1T), or 10ng/ml TGFß (10T) have the same rate of proliferation. Bars represent SEM.

CHAPTER 4

EGF INHIBITS THE ANTI-PROLIFERATIVE EFFECT OF TGFß IN PRIMARY OVARIAN CANCER CELLS

4.1 SUMMARY

TGFß inhibits proliferation of primary ovarian cancer cells in culture, which is partially mediated by up-regulation of the TGFß target gene $p15^{INK4B}$. Although primary ovarian cancer cells in culture are growth inhibited by TGFß, ovarian cancer cells *in vivo* are exposed to TGFß yet continue to proliferate. Therefore, mechanisms must exist to inhibit the anti-proliferative effect of TGFß to allow uncontrolled cellular proliferation of ovarian cancer cells. Numerous signaling pathways can converge with the TGFß signaling pathway to modulate its effects. Depending on the cell context, EGF signaling can inhibit TGFß signaling by altering R-Smad phosphorylation, R-Smad nuclear translocation, or target gene expression. The hypothesis of this study is that EGF signaling will inhibit the anti-proliferative effect of TGFß, contributing to uncontrolled cellular proliferation. Indeed, the anti-proliferative effect of TGFß is inhibited by EGF in primary ovarian cancer cells. EGF does not mediate this effect at the level of R-Smad phosphorylation or nuclear translocation, however, EGF does inhibit the TGFß induced up-regulation of $p15^{INK4B}$ expression. EGF-induced stabilization of Smad transcriptional co-repressors may mediate the inhibition of $p15^{INK4B}$ expression by TGFß. *In vivo*, the presence of growth factors such as EGF may prevent the TGFß cellular responses, contributing to the uncontrolled cellular proliferation of ovarian cancer cells. Furthermore, inhibition of these converging pathways may restore the TGFß response and provide therapeutic targets for ovarian cancer patients.

4.2 INTRODUCTION

Primary ovarian cancer cells respond to the anti-proliferative effect of TGFß (Hurteau et al. 1994; Dunfield et al. 2002). Ovarian cancer cells *in vivo* are exposed to TGFß (Abendstein et al. 2000), however these cells continue to proliferate. Therefore, mechanisms must exist to allow for uncontrolled cellular proliferation of ovarian cancer cells *in vivo*. Numerous signaling pathways can converge with the TGFß signaling pathway to modulate TGFß signaling. HGF, IFNγ, and EGF have all been shown to alter TGFß signaling in certain cell types (de Caestecker et al. 1998; Kretzschmar et al. 1999; Ulloa et al. 1999; ten Dijke et al. 2000; Funaba et al. 2002).

EGF is a mitogen, and has been shown to be mitogenic to normal OSE and ovarian cancer cells (Siemens & Auesperg, 1988; Berchuck et al. 1993; Rodriguez et al. 1991; Crew et al. 1992). The EGF receptor has been shown to be expressed in ovarian cancer (Battaglia et al. 1989; Berchuck et al. 1991; Morishige et al. 1991; Owens et al 1991b; Kohler et al. 1992) and the related receptor HER2/neu has been shown to be overexpressed in ovarian cancer and may contribute to poor prognosis (Slamon et al. 1989; Haldane et al. 1990). Because of the expression of EGF receptors in ovarian cancer, the mitogenic effect of EGF in normal OSE, and the EGF inhibition of TGFß signaling in other cell types, EGF may play a role in ovarian cancer.

This study examined whether EGF could modulate TGFß signaling *in vitro* to alter the anti-proliferative effect of TGFß and determined that EGF inhibits the anti-proliferative effect of TGFß in primary ovarian cancer cells. To further investigate this result, the effect of EGF on the different steps of the TGFß signaling pathway was examined. EGF does not inhibit TGFß signaling at the level of receptor activity, or Smad

nuclear translocation. Rather, EGF inhibits specific TGFß target gene expression. Whereas EGF does not modulate the TGFß effect on the target genes Smad7 or ID1, EGF has a synergistic effect with TGFß in increasing uPA mRNA expression. Importantly, EGF is shown to inhibit the TGFß induced up-regulation of $p15^{INK4B}$ mRNA expression, which likely contributes to the EGF inhibition of the anti-proliferative effect of TGFß in primary ovarian cancer cells.

4.3 MATERIALS AND METHODS

All methods were conducted as described in Chapter 2. Primary ovarian cancer cells were maintained in complete MCDB105/M199 medium.

4.4 RESULTS

4.4.1 EGF inhibits the anti-proliferative effect of TGFß

Previously, we have shown that TGFß can inhibit proliferation of primary ovarian cancer cells which was correlated with a TGFß induced increase in $p15^{INK4B}$ mRNA and protein production. To determine whether EGF could modulate the anti-proliferative effect of TGFß, primary ovarian cancer cells were grown in the presence of TGFß and EGF, alone and in combination, and proliferation was assessed by counting cell number. TGFß (T) (0.1 ng/ml or 1 ng/ml) inhibits proliferation of primary ovarian cancer cells, whereas EGF (E) (10 ng/ml) treated cells have a normal or higher than normal rate of proliferation compared to cells grown under normal growth conditions (UT; Figure 4-1). Cells treated with 0.1 ng/ml TGFß and 10 ng/ml EGF (ET) have a rate of proliferation significantly higher than TGFß treated cells and similar to untreated cells (UT) (Figure 4-

1A). Data is shown for OC5, OC17, OC19, OC28 and similar results were obtained for OC15, OC20, and OC23. In contrast, EGF is less effective at inhibiting the anti-proliferative effect of 1 ng/ml TGFß (OC28 and OC23) (Figure 4-1B and data not shown), however, the trend for EGF inhibition is apparent for OC17 (Figure 4-1B).

4.4.2 EGF effect on TGFß-induced Smad2 and Smad3 phosphorylation

To determine whether EGF could modulate activity of the TGFß receptor complex directly, phosphorylation of the R-Smads, Smad2 and Smad3, was determined by Western blot analysis. Results are shown for OC5 (Figure 4-2) and similar results were found for OC14-OC17 (data not shown). Phosphorylation of Smad2 (Figure 4-2A) and Smad3 (Figure 4-2B) was strongly induced by 0.1 ng/ml TGFß compared to untreated cells, whereas EGF (10 ng/ml) did not affect phosphorylation of Smad2 and Smad3. Co-treatment of cells with TGFß and EGF resulted in variable phospho-Smad2 and phospho-Smad3 levels comparable to TGFß treatment alone. In three independent experiments either no effect (Figure 4-2A) or a decrease in Smad2 phosphorylation (Figure 4-2C) was observed in co-treated compared to TGFß treated cells, and this differential Smad phosphorylation was seen in the same cells of identical passage number. A complete blockade of Smad2 phosphorylation was never observed. Smad3 phosphorylation was typically unaffected by co-treatment (Figure 4-2B), although slight decreases were observed in one experiment for two of the five ovarian cancer samples.

Although it appears that total Smad2 levels are increased by TGFß treatment (Figure 4-2A), this effect is due to the ability of the total Smad2 antibody to recognize both phosphorylated and unphosphorylated forms of Smad2, which can be seen when the

2 forms of Smad2 are further resolved (Figure 4-2C). A similar effect was observed for Smad3 (data not shown).

4.4.3 EGF does not affect TGFß-induced nuclear localization of Smad2 and Smad3

Post-receptor activation of R-Smads leads to nuclear translocation, therefore, immunofluorescence was done to determine if EGF affected the TGFß-induced nuclear translocation of Smad2 and Smad3 in primary ovarian cancer cells. Cellular localization of Smad2 and Smad3 in OC24 is shown (Figure 4-3A and 4-3B, respectively), and similar results were found with OC5, OC15, and OC32. Untreated cells have diffuse nuclear and cytoplasmic localization of Smad2 and Smad3, however, treatment with TGFß induces Smad2/3 nuclear translocation (Figure 4-3). Treatment with EGF has no observable effect on Smad nuclear translocation and co-treatment resulted in Smad nuclear translocation comparable to cells treated with TGFß alone.

4.4.4 EGF effect on TGFß target gene expression

EGF does not modulate the TGFß-induced up-regulation of Smad7 mRNA: Smad7 transcription is up-regulated in response to TGFß signaling (Nakao et al. 1997b). In order to determine whether EGF could modulate the TGFß response at the transcriptional level, Northern analysis was done to examine Smad7 mRNA expression in OC15 – OC17 (Figure 4-4A). Untreated samples express low levels of Smad7 mRNA, which is induced by TGFß treatment. EGF has been shown to up-regulate Smad7 mRNA in mink lung epithelial cells (Afrakhte et al. 1998), however, EGF treatment either has no effect or only slightly increases Smad7 mRNA expression in primary ovarian cancer cells. Co-treatment with EGF does not cause a decrease in TGFß-induced Smad7 mRNA

levels. GAPDH mRNA expression was used as a loading control for normalization of the signal.

EGF synergistically increases TGFß-induced uPA mRNA expression: uPA expression can be increased or decreased in response to TGFß, depending on cell type (Lund et al. 1987; Kieki-Oja et al. 1988b). Previous work from this lab has shown TGFß increased uPA expression in ovarian cancer cell lines (Dr. Y.Fu; personal communication). EGF has also been shown to up-regulate uPA expression (Grimaldi et al. 1986; Niedbala et al. 1989). Primary ovarian cancer cells (OC15-OC17) have a slight increase in uPA mRNA expression in response to TGFß, and EGF enhances uPA mRNA expression (Figure 4-4B). Interestingly, uPA mRNA expression is synergistically up-regulated in response to TGFß and EGF co-treatment.

EGF has no effect of TGFß-induced down-regulation of ID1 mRNA expression: Ovarian cancer cell lines up-regulate inhibitor of differentiation 1 (ID1) mRNA expression in response to TGFß (Dr. T. Shepherd, personal communication), however, the basal level of ID1 mRNA in untreated primary ovarian cancer cells is decreased with TGFß treatment in OC5, OC15, OC16 (Figure 4-4C). EGF has no effect on ID1 mRNA expression alone, and levels of ID1 mRNA in TGFß and EGF co-treated primary ovarian cancer cells is the same compared to that of TGFß treatment alone.

4.4.5 EGF modulates TGFß-induced $p15^{INK4B}$ mRNA expression

Since EGF can abrogate the anti-proliferative effect of TGFß, we examined whether EGF could modulate TGFß-induced expression of $p15^{INK4B}$ mRNA. $p15^{INK4B}$ mRNA expression is shown for OC15 (Figure 4-5), and similar results were found for OC5, OC14, OC16 and OC17 (data not shown). Untreated samples of asynchronously

growing cells express little or no $p15^{INK4B}$ mRNA, whereas TGFß strongly induces $p15^{INK4B}$ mRNA expression (Figure 4-5). EGF alone has no effect on $p15^{INK4B}$ mRNA levels, however, co-treatment of EGF with TGFß reduces $p15^{INK4B}$ mRNA levels below that of TGFß treatment alone. GAPDH signal was used to control for loading. Normalized data is shown in Figure 4-5B, and the expression levels and percent reduction (39.1% - 61.4%) for co-treated samples compared to TGFß treated samples are shown in Figure 4-5C.

4.5 DISCUSSION

Modifications to TGFß signaling have been indicated in numerous types of cancers, including ovarian (Lynch et al. 1999; Wang et al. 2000; Chen et al. 2001), however, we previously found that the TGFß signaling pathway was intact and functional in primary ovarian cancer cells (Dunfield et al. 2002). Since ovarian cancer cells produce and are exposed to TGFß (Abendstein et al. 2000; Auersperg et al. 2001), inhibition of the anti-proliferative effect of TGFß must exist to allow for the uncontrolled cellular proliferation of ovarian cancer cells *in vivo*. We show that EGF inhibits the anti-proliferative effect of TGFß in primary ovarian cancer cells, and suggest that it may be one factor of many that can modulate TGFß cellular responses *in vivo*.

Alterations in TGFß signaling by EGF and related factors have been demonstrated in a number of studies, examining modulation of TGFß-induced R-Smad nuclear translocation by EGF, HGF or oncogenic ras. Studies by Kretzschmar et al. (1999) have shown that oncogenic ras and EGF can phosphorylate residues in the linker region of Smad2 and Smad3 (Kretzschmar et al. 1999), which results in inhibition of TGFß-

induced Smad nuclear translocation in mouse mammary epithelial cells. Studies by de Caestecker et al. (1998) show that HGF can phosphorylate Smad2, resulting in increased Smad2 nuclear translocation in a breast cancer cell line, and further speculate that EGF will have the same effect (de Caestecker et al. 1998). In contrast, we find that EGF does not affect TGFß-induced Smad2 or Smad3 nuclear translocation in primary human ovarian cancer cells (Figure 4-3), suggesting that crosstalk between the TGFß and EGF signaling pathways may be cell type dependent.

In order for R-Smads to translocate to the nucleus, they must be phosphorylated by an activated receptor complex. We found that EGF co-treatment did not result in loss of R-Smad phosphorylation (Figure 4-2) which is in agreement with our results that show R-Smad nuclear translocation is unaffected by EGF co-treatment. Because decreased levels of phosphorylated Smad2 and Smad3 were occasionally observed with EGF co-treatment, the possibility that EGF can modulate TGFß signaling at the receptor level cannot be ruled out. Our data strongly suggests however, that EGF has minimal effects on TGFß signaling at the level of R-Smad phosphorylation in ovarian cancer cells. Furthermore, complete blockade of TGFß-induced R-Smad phosphorylation by EGF was never observed.

Smad7 is an inhibitory Smad protein that can negatively regulate the TGFß pathway by inhibiting R-Smad phosphorylation by the activated receptor complex (Hayashi et al. 1997; Nakao et al. 1997b), and it has been shown that EGF can up-regulate expression of Smad7 in mink lung epithelial cells (Afrakhte et al. 1998). In comparison to the strong induction of Smad7 mRNA expression in response to TGFß, we observed minimal induction of Smad7 mRNA by EGF in primary ovarian cancer cells,

and thus, it is unlikely that EGF induces a negative effect on TGFß signaling via Smad7 (Figure 4-4A). Indeed, our results show that EGF co-treatment does not affect TGFß signaling activity from the receptor to the nucleus. In addition to Smad7, TGFß effects on Id1 were also not modulated by EGF. Id genes are up-regulated by BMP (Ogata et al. 1993), and Id1 has been shown to be up-regulated by TGFß in ovarian cancer cell lines (Dr. T. Shepherd, personal communication). In contrast, TGFß decreases Id1 mRNA expression in primary ovarian cancer cells, and this was not modulated by EGF co-treatment (Figure 4-4C). Conversely, uPA is both a TGFß and an EGF target gene (Grimaldi et al. 1986; Lund et al. 1987; Keski-Oja et al. 1988; Niedbala et al. 1989). Whereas EGF strongly induced uPA mRNA expression, TGFß only slightly up-regulated uPA mRNA expression. Interestingly, EGF and TGFß co-treatment resulted in a synergistic increase in uPA mRNA expression (Figure 4-4B). The uPA promoter contains Sp1 and AP1 sites (Nerlov et al. 1991; Nerlov et al. 1992; Ibanez-Tallon et al. 2002). Smads up-regulate other TGFß target genes that contain these sites by association with Sp1 and AP-1 transcription factors, and EGF can induce AP-1 expression. Therefore, the synergistic increase in response to EGF and TGFß co-treatment may reflect the need for association of AP-1, Sp1 and Smads at the uPA promoter in order to efficiently induce transcription. Overall, since EGF modulation of TGFß-induced target gene expression is clearly not a general effect, but rather, specific to the target genes, this particularly implies that EGF modulation of TGFß signaling does not occur prior to target gene expression.

In studies of TGFß and EGF on normal bovine OSE cells, Vigne et al. (1994) found that EGF stimulated proliferation of bovine OSE, and this effect was partially

decreased by TGFß co-treatment (Vigne et al. 1994). They did not observe an effect of TGFß alone on bovine OSE proliferation and suggest that TGFß inhibits the mitogenic effect of EGF. These results were obtained using a [^{3}H]-thymidine incorporation assay after 24h of treatment. The lack of a TGFß response may be due to the short time course of treatment or possibly species differences. In the present study, primary ovarian cancer cells were treated and counted over a period of 7 days, and the maximal responses were seen at the later time points. Although the possibility exists that TGFß inhibits the mitogenic effect of EGF, our observation that EGF inhibits the TGFß induced up-regulation of $p15^{INK4B}$ mRNA expression strongly suggests that EGF inhibits the anti-proliferative effect of TGFß in human ovarian cancer cells.

EGF can inhibit the anti-proliferative effect of 0.1 ng/ml TGFß in all primary ovarian cancer cells tested (n=7; Figure 4-1A), however, inhibition of 1 ng/ml TGFß by EGF is less consistent (Figure 4-1B). This is not surprising, considering other studies have shown differential effects of TGFß and EGF on Smad nuclear translocation in mink lung epithelial cells depending on the dose of TGFß (Kretzschmar et al. 1999). Although a trend for EGF inhibition of the anti-proliferative effect of 1 ng/ml TGFß was seen in primary ovarian cancer cells, this effect was not statistically significant, and appeared to be cell sample and passage number dependent. Although a maximal dose of EGF (10 ng/ml) was used in the present study, 1 ng/ml EGF inhibited the anti-proliferative effect of TGFß at the same level compared to 10 ng/ml EGF. Higher doses of EGF were not tested to determine whether EGF could fully inhibit the anti-proliferative effect of TGFß.

Although we show that EGF can partially inhibit the anti-proliferative effect of TGFß in ovarian cancer cells, other signaling pathways may also contribute to abrogation of the TGFß signal. HGF and oncogenic ras have been shown to alter TGFß signaling in human breast cancer cells and mouse mammary epithelial cells, respectively (de Caestecker et al. 1998; Kretzschmar et al. 1999). In addition, IFNγ can inhibit nuclear translocation of Smad3 in a mutant human fibrosarcoma cell line (Ulloa et al. 1999), which could interfere with TGFß transcriptional responses. These factors may also be present and act to inhibit TGFß signaling *in vivo*, contributing to uncontrolled cellular proliferation. Indeed, HGF was found to be present in ascites fluid of ovarian cancer patients (Sowter et al. 1999).

EGF inhibits TGFß-induced $p15^{INK4B}$ mRNA expression, which likely accounts for the EGF inhibition of the anti-proliferative effect of TGFß in primary ovarian cancer cells. A possible mechanism for EGF inhibition of the TGFß induced up-regulation of $p15^{INK4B}$ expression is via EGF stabilization of the Smad transcriptional co-repressor TGIF (Figure 4-6). EGF signaling phosphorylates TGIF, resulting in TGIF stabilization and interaction with Smad2 (Lo et al. 2001). TGIF binding to Smad2 induces formation of a complex at TGFß target gene promoters and inhibition of gene expression through recruitment of histone deacetylases (HDACs) (Wotton et al. 1999a). Indeed, over-expression of TGIF in HaCaT cells has been shown to inhibit $p15^{INK4B}$ mRNA expression (Lo et al. 2001). We found that TGIF mRNA is expressed in primary ovarian cancer cells (Figure 4-7). Future work could examine whether stabilization of TGIF results in recruitment of HDAC to the $p15^{INK4B}$ promoter and inhibition of TGFß induced $p15^{INK4B}$ mRNA expression in primary ovarian cancer cells.

Although the TGFß signaling pathway is intact in primary ovarian cancer cells, mechanisms must exist to alter cellular responses to TGFß and contribute to uncontrolled cellular proliferation. We show that EGF hinders the TGFß-induced up-regulation of $p15^{INK4B}$, and this likely contributes to inhibition of the anti-proliferative effect of TGFß in primary ovarian cancer cells. *In vivo*, the presence of additional growth factors may prevent TGFß cellular responses, and it is likely a combination of factors that act to modulate cellular responses. Inhibition of these signaling pathways may therefore help to restore the anti-proliferative effect of TGFß, and therapeutics that block these other pathways may be important treatment options for ovarian cancer patients.

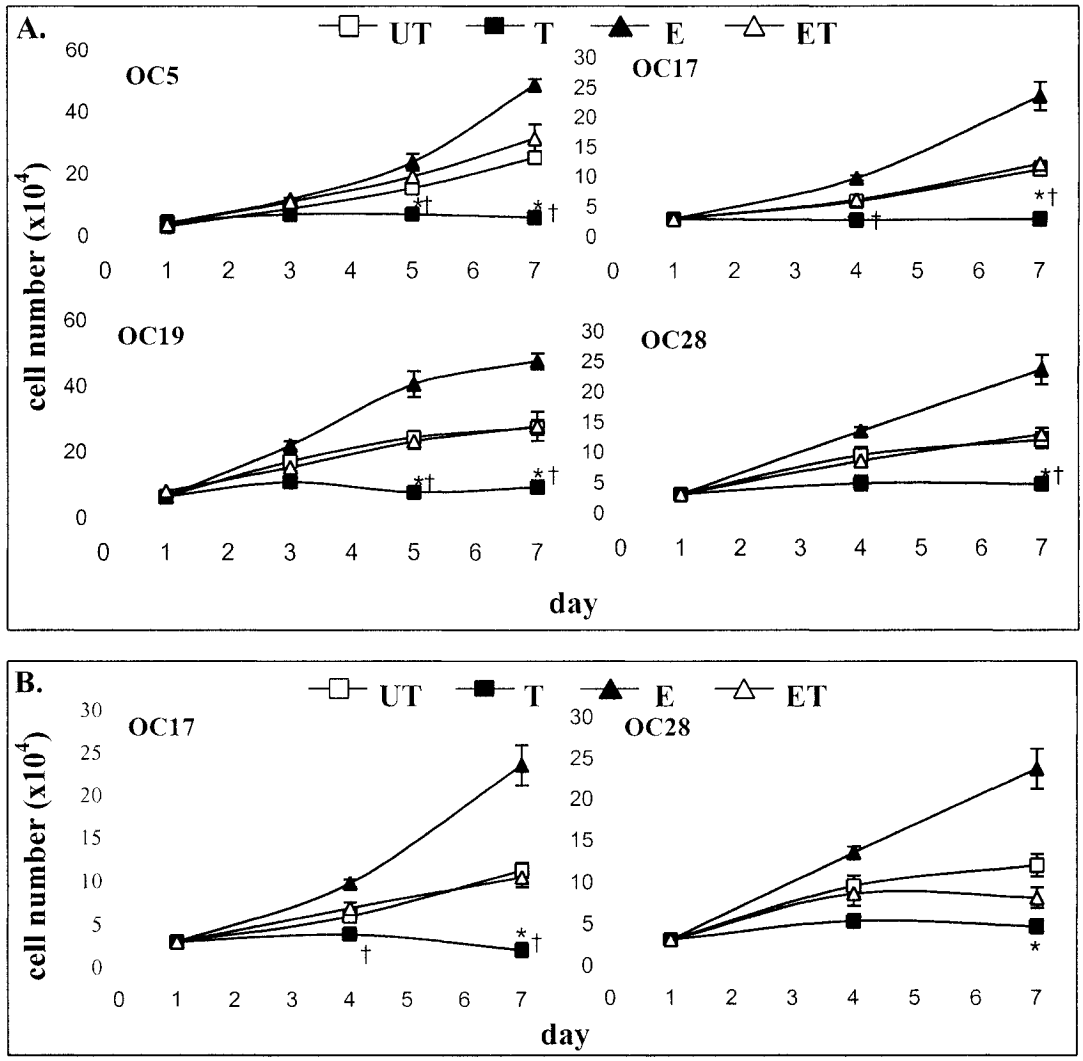


Figure 4-1. EGF inhibits the anti-proliferative effect of TGFß A) Primary ovarian cancer cells (OC) treated with 0.1ng/ml TGFß (T) are growth inhibited. EGF (10ng/ml) treated cells (E) have a normal or slightly higher than normal rate of proliferation compared to cells grown under normal growth condition (UT). The rate of proliferation of cells treated with EGF and TGFß (ET) have a rate of proliferation significantly higher than that of TGFß treated cells. **B)** Primary ovarian cancer cells treated with 1ng/ml TGFß are also growth inhibited. EGF is less effective at inhibiting the anti-proliferative effect of 1ng/ml TGFß (ET). The graphs show representative data from three separate experiments. Bars represent SEM. (* = significant difference between untreated and TGFß treated cells; † = significant difference between TGFß treated compared to EGF and TGFß co-treated cells; $P<0.05$)

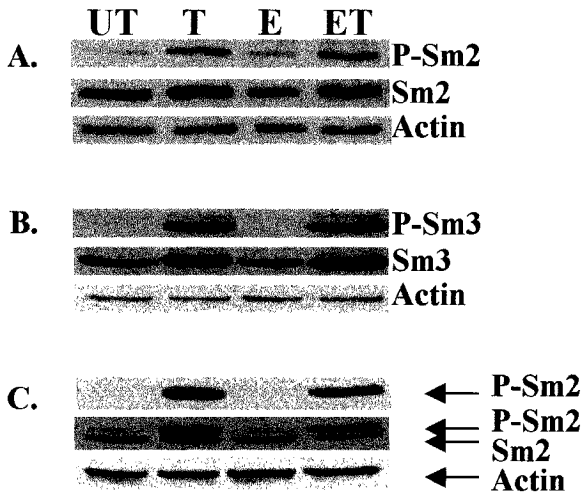


Figure 4-2: EGF effect on TGFß-induced Smad2 and Smad3 phosphorylation. Untreated (UT) primary ovarian cancer cells (OC5) have little or no phosphorylated Smad2 (P-Sm2) or Smad3 (P-Sm3) protein. TGFß (T) strongly induces phosphorylation of both Smad2 **(A)** and Smad3 **(B)**. EGF (E) alone does not affect levels of Smad2 or Smad3 phosphorylation, whereas cells co-treated with TGFß and EGF (ET) have similar levels of phosphorylated Smad2 and Smad3 compared to cells treated with TGFß alone. The apparent increase in total Smad2 levels in response to TGFß treatment **(A)** is actually a result of the total Smad2 antibody recognizing both phosphorylated and non-phosphorylated forms of Smad2, which can be seen when the proteins are further resolved **(C;** middle panel**)**. Actin protein levels were assessed to control for loading.

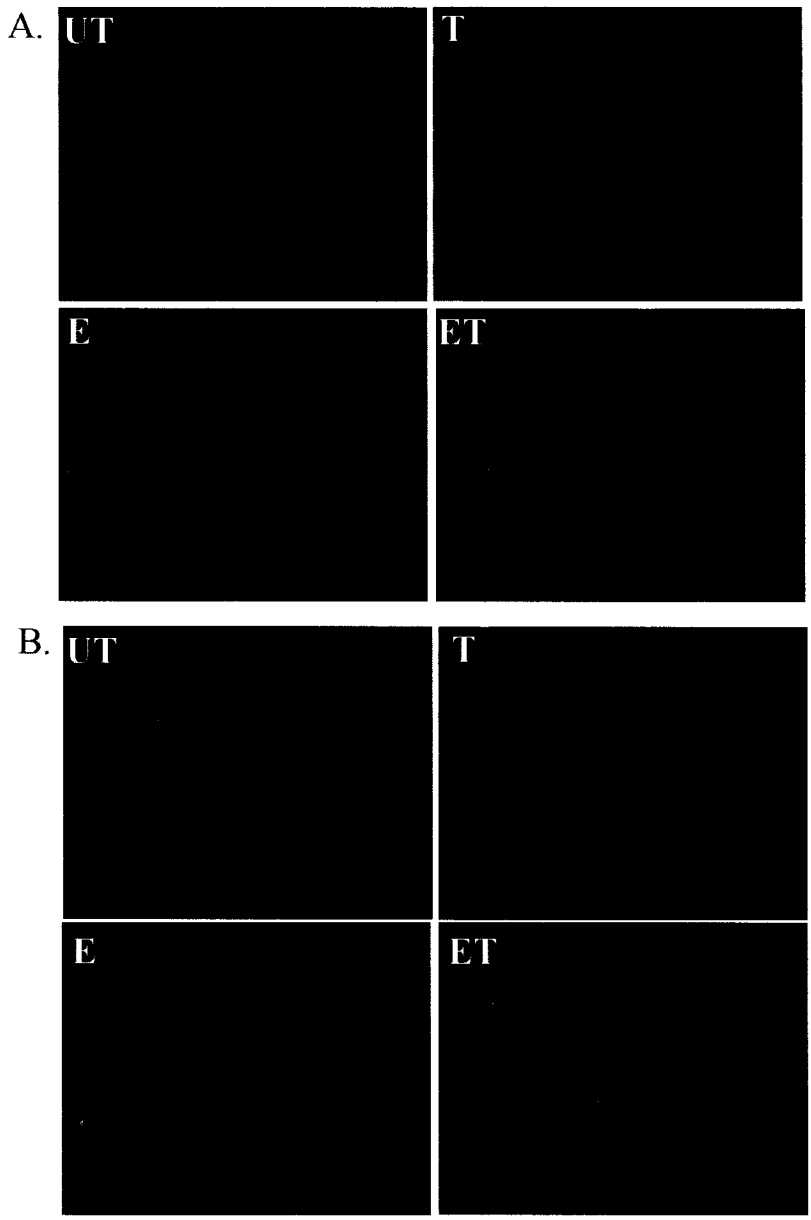


Figure 4-3: EGF does not affect TGFß induced R-Smad nuclear translocation. Untreated (UT) primary ovarian cancer cells (OC24) have diffuse nuclear and cytoplasmic localization of Smad2 **(A)** and Smad3 **(B).** Treatment with TGFß (T) induces R-Smad nuclear translocation, and this is not inhibited by co-treatment with EGF (ET). EGF treatment (E) alone does not induce Smad2 or Smad3 nuclear translocation.

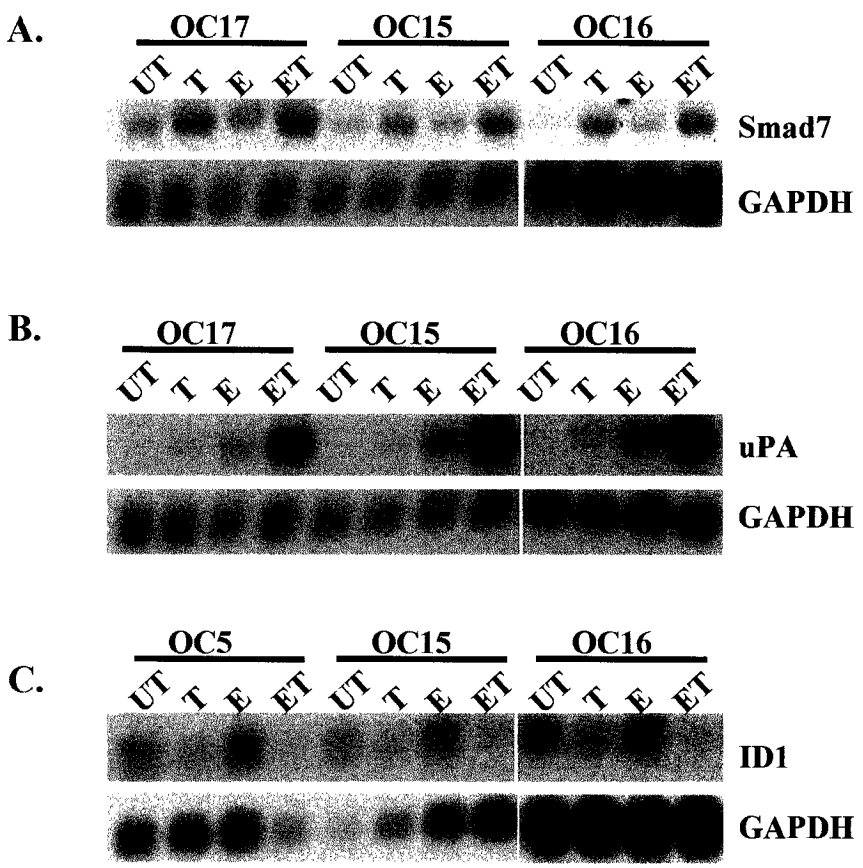
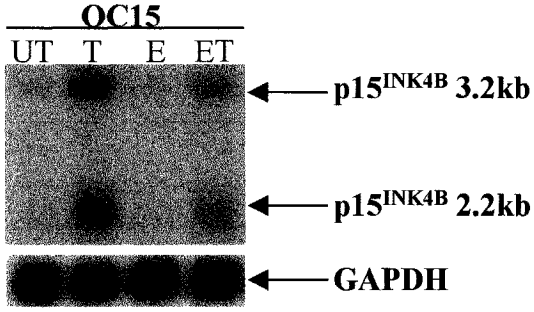
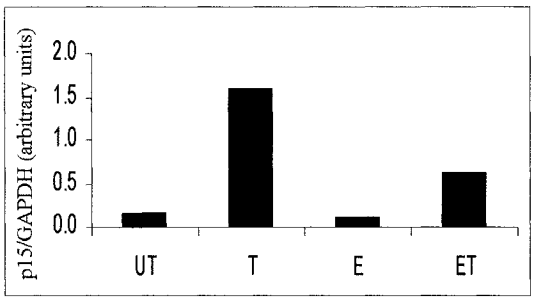


Figure 4-4: EGF effect on TGFß target gene expression. A) Untreated (UT) primary ovarian cancer cells exhibit basal Smad7 mRNA expression which is increased by TGFß treatment (T). EGF (E) alone has little effect, and EGF co-treatment (ET) does not inhibit TGFß-induced Smad7 mRNA levels. **B)** TGFß only slightly increases uPA mRNA expression, whereas EGF increases uPA mRNA expression. EGF and TGFß co-treatment results in a synergistic increase in uPA mRNA expression. **C)** Basal ID1 mRNA expression is decreased by treatment with TGFß, and EGF has no effect. TGFß and EGF co-treated cells have the same ID1 mRNA expression compared to TGFß treatment alone. GAPDH mRNA levels were assessed to control for loading.

A.



B.



C.

	Fold expression			Percent Reduction
	T	E	ET	
OC5	9.8	1.4	6.0	39.1
OC14	4.2	0.9	2.2	47.4
OC15	9.6	0.6	3.7	61.4
OC16	3.6	0.4	1.9	47.3
OC17	11.5	1.1	5.4	53.1

Figure 4-5: EGF inhibits the TGFß induced up-regulation of $p15^{INK4B}$. OC15 cells were either untreated (UT), treated with TGFß (T), or EGF (E), alone or in combination (ET). **A)** Untreated cells and cells treated with EGF have little or no $p15^{INK4B}$ mRNA expression (3.2kb and 2.2kb mRNA). TGFß induces $p15^{INK4B}$ expression, whereas co-treatment with EGF reduces levels of TGFß-induced $p15^{INK4B}$ mRNA expression. **B)** Quantification of the $p15^{INK4B}$ signal shown in (A) was done by densitometry of the 3.2kb mRNA signal and normalized to GAPDH mRNA. **C)** Expression levels and percent reduction of $p15^{INK4B}$ mRNA in co-treated compared to TGFß treated cells.

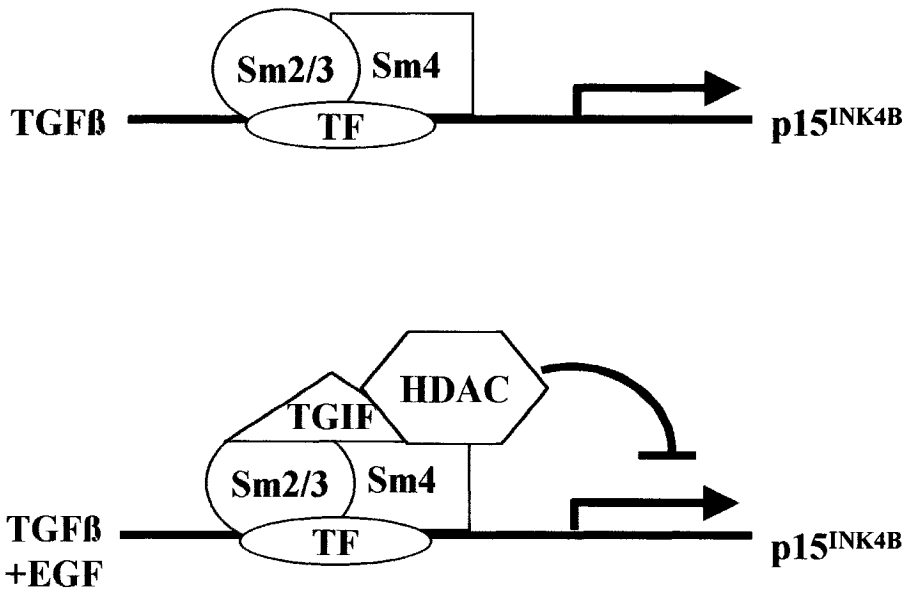


Figure 4-6: Proposed mechanism of EGF inhibition of the TGFß induced up-regulation of p15 mRNA expression. TGFß signaling results in $p15^{INK4B}$ mRNA transcription via Smad2/3 (Sm2/3) and Smad4 (Sm4) binding to the $p15^{INK4B}$ promoter in association with other transcription factors (TF). EGF may cause recruitment of the Smad transcriptional co-repressor TGIF, which recruits histone deacetylase (HDAC). HDAC protein could then modify chromatin structure to turn off $p15^{INK4B}$ mRNA expression.

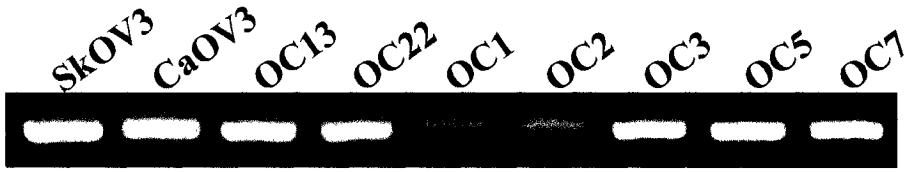


Figure 4-7: TGIF mRNA expression in ovarian cancer cell lines and primary ovarian cancer cells. RT-PCR using cDNA from SkOV3 and CaOV3 cells, and OC1-3, OC5, OC7, OC13, and OC22 show expression of TGIF mRNA.

CHAPTER 5

CONCENTRATION AND ACTIVITY OF TGFß AND EGF IN ASCITES FLUID

5.1 SUMMARY

Although studies have demonstrated that TGFß and EGF have effects on normal OSE as well as ovarian cancer cells, the concentration of these growth factors in the ascites fluid of ovarian cancer patients is largely unknown. TGFß concentrations have been measured in peritoneal fluid of ovarian cancer patients, however, it is unknown whether active or total TGFß was measured. The objective of this study was to determine the concentration of TGFß and EGF in ascites fluid. Immunoassays were used to measure levels of total (active + latent) and active TGFß, as well as EGF in clarified ascites samples from ovarian cancer patients. Active TGFß ranged from 1.5 – 3.5 ng/ml, with an average of 2.3 ng/ml and total TGFß levels ranged from 4.7 – 12.4 ng/ml, with an average of 7.3 ng/ml. Functional analysis of the active TGFß present in ascites fluid revealed that the TGFß present in ascites fluid is able to signal, however, signaling activity is much lower than what would be expected if all TGFß present in ascites was able to signal. Therefore, only a fraction of the TGFß present is actually able to transmit a TGFß signal, and hence must be inhibited prior to activation of the signaling pathway. EGF was also measured in ascites fluid, and found to range from only 23 – 38.8pg/ml, with an average of 30.5pg/ml. Although EGF levels were low, other factors that signal in a manner similar to EGF could account for inhibition of TGFß signaling. The remaining TGFß signal may therefore be inhibited by EGF or factors that signal in a similar manner resulting in an overall inhibition of the anti-proliferative effect of TGFß.

5.2 INTRODUCTION

Ascites is the accumulation of fluid in the peritoneum, which can be a complication of late stage gynecologic cancers (Hirabayashi and Graham 1970; Stratton and DiSaia 1981). The ascites fluid is produced by non-cancerous surfaces of the peritoneum (Hirabayashi and Graham 1970) and contains tumor cells, serum protein, mononuclear cells, and macrophages (Stratton and DiSaia 1981). Purification of tumor cells from ascites fluid is a common method of isolating primary ovarian cancer cells from late stage ovarian cancer patients (Hirte et al. 1992).

The levels of TGFß present in ascites fluid were assessed by Abendstein et al. (2000), and found to have a mean concentration of 5.443 ng/ml (range 0.737 ng/ml – 14.687 ng/ml). TGFß is secreted as a latent precursor, which must be activated in order to bind the type II TGFß receptor (Assoian et al. 1983; Miyazono et al. 1988; Wakefield et al. 1988; Wakefield et al. 1990). The Abendstein study did not distinguish between active and latent TGFß, therefore, their measurements may include both active and latent TGFß. An immunoassay utilizing the type II TGFß receptor was used in the present study to determine the concentration of active TGFß present in ascites fluid. Furthermore, assays were done to examine TGFß signaling activity of ascites fluid. The mean level of active TGFß was found to be 2.3 ng/ml, however, the activity of this TGFß in ascites fluid is lower than what is expected if all active TGFß present in ascites fluid is able to transmit the signal. Levels of EGF in ascites fluid were also assessed in this study.

5.3 MATERIALS AND METHODS

All methods were conducted as described in Chapter 2. Ascites fluid samples were clarified by centrifugation (15 min at 4000 rpm) at the time the samples were obtained, and the supernatants were stored at -80°C.

5.4 RESULTS

5.4.1 Concentration of TGFß and EGF in ascites fluid

To measure the concentration of active TGFß and EGF in ascites fluid, commercial enzyme linked immunosorbent assays (ELISAs) were used. Active TGFß ranged from 1.5 – 3.5 ng/ml, with an average of 2.3 ng/ml (Table 5-1A). Total TGFß was also measured by activating the latent TGFß that may be present in ascites fluid through an acidification process. The concentration of total TGFß ranged from 4.7 ng/ml - 12.4 ng/ml, with an average of 7.3 ng/ml. EGF levels in ascites fluid ranged from 23 pg/ml – 38.8 pg/ml, with an average of 30.5 pg/ml (Table 5-1B).

5.4.2 Ascites fluid does not inhibit DNA synthesis

[^{3}H]-thymidine incorporation in response to ascites fluid was assessed to determine if ascites fluid (0.1 ng/ml – 0.5 ng/ml ascitic TGFß) could inhibit DNA synthesis in the TGFß-sensitive mink lung epithelial (Mv1Lu) cells. Inconsistent results were observed for 3 independent experiments when cells were treated with ascites fluid, however, a decrease in DNA synthesis was not seen, with the exception of ascites samples A24 and A25 (Figure 5-1A).

5.4.3 TGFß in ascites fluid does not inhibit DNA synthesis

A neutralizing antibody against TGFß was used to determine whether it is the TGFß present in ascites samples A24 and A25 that inhibits DNA synthesis in Mv1Lu cells. The decrease in [^{3}H]-thymidine incorporation in response to A24 and A25 (0.5 ng/ml ascitic TGFß) was not affected by co-treatment with neutralizing antibody (25 µg/ml; Figure 5-1B). Initial experiments were done to confirm blocking ability of the antibody against TGFß, and found the inhibition of DNA synthesis in response to TGFß in Mv1Lu cells was completely abolished by co-treatment with neutralizing antibody.

5.4.4 Exogenous TGFß is inhibited by ascites fluid

Co-treatment of Mv1Lu cells with ascites samples and recombinant TGFß was done to assess the possibility that a component in ascites fluid can inhibit exogenously added TGFß. Treatment with TGFß (0.5 ng/ml) caused a decrease in [^{3}H]-thymidine incorporation (Figure 5-1C). Ascites fluid had little effect alone and co-treatment of cells with ascites fluid and TGFß (0.1 ng/ml – 5 ng/ml) resulted in similar levels of [^{3}H]-thymidine incorporation compared to treatment with ascites fluid alone.

5.4.5 Ascites fluid induces Smad2 phosphorylation

SkOV3 cells were treated with ascites fluid (0.74 ng/ml – 1.73 ng/ml ascitic TGFß) and total cellular protein was analyzed by Western blot to determine whether ascites fluid induces Smad2 phosphorylation. Western analysis indicates an increase in phosphorylation of Smad2 in response to treatment of SkOV3 cells with ascites fluid, however, the level of Smad2 phosphorylation is lower compared to cells treated with 0.1 ng/ml recombinant TGFß (Figure 5-2).

5.4.6 TGFß in ascites fluid induces Smad2 phosphorylation

SkOV3 cells were treated with ascites fluid and TGFß neutralizing antibody to determine whether it is the TGFß present in ascites fluid that is responsible for the phosphorylation of Smad2. The increase in Smad2 phosphorylation seen with ascites fluid treatment is inhibited by co-treatment with TGFß neutralizing antibody (Figure 5-3).

5.5 DISCUSSION

The concentration of active TGFß in ascites fluid was measured by immunoassay. Previous studies have measured TGFß in ascites fluid and found the concentration of TGFß in ascites fluid to be 5.4 ng/ml, however, this study did not distinguish between active and latent TGFß (Abenstein et al. 2000). By using an immunoassay that utilizes the type II TGFß receptor to recognize TGFß, active TGFß can be measured. We found the concentration of active TGFß to range from 1.5 ng/ml to 3.5 ng/ml, with an average of 2.3 ng/ml (Table 5-1A). Acidification of ascites fluid was done to activate any latent TGFß present in order to measure total (active + latent) TGFß. Total TGFß concentrations ranged from 4.7 ng/ml to 12.4 ng/ml, with an average of 7.3 ng/ml.

To examine the signaling activity of TGFß in ascites fluid, DNA synthesis and phosphorylation of Smad2 in response to ascites fluid were examined. Mv1Lu cells were used to assess DNA synthesis, since these cells are known to respond to the anti-proliferative effect of TGFß which has been measured using [^{3}H]-thymidine incorporation assays (Cheifetz et al. 1987; Boyd and Massague 1989). Mv1Lu cells were treated with ascites fluid, and DNA synthesis was measured using [^{3}H]-thymidine incorporation assays. It was expected that the TGFß present in ascites fluid would inhibit

DNA synthesis in Mv1Lu cells, however, no inhibition of DNA synthesis occurred in response to ascites fluid (Figure 5-1A). Although this may indicate that the TGFß present in ascites fluid is not active, it may simply be inhibition of TGFß activity by another factor also present in ascites fluid. Results from the [^{3}H]-thymidine assays treating Mv1Lu cells with ascites fluid were inconsistent. The initial experiment showed an increase in DNA synthesis in response to ascites fluid. Additional growth factors present may enhance DNA synthesis in Mv1Lu cells, which may be masking an anti-proliferative response of TGFß. The proliferative response to ascites fluid in the first experiment was lost in the later experiments, which may be due to loss of activity of some components in ascites fluid, such as growth factors, related to repeated freeze thaw cycles or extended storage time.

The exception in the DNA synthesis assays is A24 and A25, which inhibited DNA synthesis in all 3 experiments. Although A24 and A25 consistently inhibit DNA synthesis in Mv1Lu cells, neutralizing antibody against TGFß did not reverse this effect (Figure 5-1B). Therefore, the TGFß present in ascites fluid is not responsible for the inhibition of DNA synthesis, but rather another component present in ascites fluid.

TGFß is known to inhibit DNA synthesis in Mv1Lu cells and treatment of Mv1Lu cells with recombinant TGFß inhibits DNA synthesis in this study; however, this effect is blocked by treatment with ascites fluid (Figure 5-1C). This result suggests that there may be components present in ascites fluid that can inhibit TGFß. The potential presence of these components may also account for the lack of inhibition of DNA synthesis in response to the TGFß present in ascites fluid.

The activity of TGFß in ascites fluid was also tested by examining the phosphorylation status of Smad2 in SkOV3 cells treated with ascites fluid. Treatment of SkOV3 cells with ascites fluid (0.74 ng/ml – 1.73 ng/ml ascitic TGFß) results in phosphorylation of Smad2 (Figure 5-2). Because other components are present in ascites fluid which may induce Smad2 phosphorylation, neutralizing antibodies against TGFß were used to confirm that it is the TGFß present in ascites fluid that is responsible for phosphorylation of Smad2. The phosphorylation of Smad2 in response to ascites fluid was reversed by co-incubation with TGFß neutralizing antibody, indicating this Smad2 phosphorylation is indeed due to the TGFß present in ascites fluid (Figure 5-3). The level of Smad2 phosphorylation, however, is lower than what is expected if all TGFß present in ascites fluid is able to signal. A concentration of 0.1 ng/ml recombinant TGFß is able to strongly induce Smad2 phosphorylation, however, a greater concentration of TGFß in ascites fluid (1.5 ng/ml) results in lower levels of Smad2 phosphorylation. This response, however, was greater in the earlier experiments (Figure 5-2) compared to a later experiment (Figure 5-3) even though higher concentrations of ascitic TGFß was used in the later experiment (Figure 5-3). Like the DNA synthesis experiments, this decreased response may be due to repeated freeze thaw cycles and prolonged storage time, resulting in inactivation of components present in ascites fluid. In addition, cells were incubated in a total volume of 4 ml. The addition of higher ascitic TGFß levels causes the amount of medium used in these experiments to be lower, thus, the cells may be less responsive due to alterations in the cellular environment. In general, although some TGFß is able to transmit a TGFß signal, there is inhibition of TGFß signaling activity in ascites fluid, possibly by other factors acting to inhibit TGFß receptor activity.

The level of EGF in ascites fluid was determined to range from 23 pg/ml to 38.8 pg/ml, with an average of 30.5 pg/ml. Although low levels of EGF were detected in ascites fluid, EGF signaling may still affect the anti-proliferative effect of TGFß, via ligand independent signaling associated with overexpression of HER2/neu, or alternative ligand binding to the EGF receptor. HER2/neu is a member of the EGF family of receptors, and can heterodimerize with the EGF receptor. Overexpression of HER2/neu results in ligand independent signaling, and has been correlated with poor prognosis in breast and ovarian cancer (Slamon et al. 1989; Haldane et al. 1990). HER2/neu receptor is overexpressed in over 30 % of ovarian cancers, and thus, may result in EGF signaling events that may contribute to inhibition of TGFß signaling. In addition, TGFα signals through the EGF receptor. TGFα is expressed in ovarian cancer cell lines, as well as normal, benign and malignant ovarian samples and has been shown to induce proliferation of primary human ovarian cancer cells (Morishige et al. 1991) and ovarian cancer cell lines (Crew et al. 1992; Stromberg et al. 1992). Therefore, TGFα may contribute to inhibition of the anti-proliferative effect of TGFß in ovarian cancer *in vivo*. Although TGFα was not measured in this study, other studies have examined and detected TGFα in ovarian cancer cell lines, primary ovarian cancer cells, and ovarian tumor samples (Morishige et al. 1991; Owens et al. 1991a; Kohler et al. 1992; Burke et al. 1996; Chien et al. 1997; Saltzman et al. 1999). Saltzman et al. measured TGFα levels in serum and peritoneal fluid of ovarian cancer patients. Serum levels of TGFα were found in half of the ovarian cancer patients, however, only 6/36 of the ascites samples contained detectable levels of TGFα (Saltzman et al. 1999). Although detectable TGFα

(10.2 pg/ml – 24.7 pg/ml) was only found in a small number of ascites samples, TGFα may contribute to inhibition of TGFß signaling.

Oncogenic ras may also contribute to the inhibition of the anti-proliferative effect of TGFß *in vivo*. Metastasis and invasion induced by TGFß is dependent on the presence of oncogenic ras (Janda et al. 2002). Furthermore, ras transformed cells are growth stimulated by TGFß, whereas the normal and non-metastatic counterparts are growth inhibited by TGFß (Schwarz et al. 1988). Activating ras mutations have been implicated in mucinous ovarian carcinomas (Enomoto et al. 1990; Enomoto et al. 1991; Garrett et al. 2001) and therefore, ras activation may contribute to inhibition of the anti-proliferative effect of TGFß as well as metastasis and invasion in ovarian cancer.

Although high concentrations of active TGFß were found to be present in ascites fluid, there is inhibition of TGFß in ascites fluid. The increase in Smad2 phosphorylation in response to ascites is inhibited by neutralizing antibody against TGFß and therefore, TGFß present in ascites fluid is able to signal. The level of phosphorylation, however, is much lower than what is expected if all TGFß present in ascites fluid is able to signal, therefore, other factors present in ascites fluid likely cause inhibition of TGFß signaling activity. In addition, the residual signaling induced by TGFß may be inhibited by other factors present in ascites fluid, such as TGFα and EGF, resulting in an overall inhibition of the anti-proliferative effect of TGFß.

A.

		conc (ng/ml)	
ascites #	**OC #**	**active**	**total**
A15	OC15	1.7	9.6
A16	OC16	2.3	4.8
A21	OC21	1.6	12.4
A22	OC22	2.8	7.7
A23	OC23	1.5	7.5
A24	OC24	3.1	7.8
A25	OC25	1.6	7.0
A26	OC26	3.0	4.7
A28	OC28	1.8	9.0
A30	OC30	3.0	6.0
A32	OC32	3.5	5.8
	AVG	2.4	7.3

B.

ascites #	**OC #**	**conc (pg/ml)**
A15	OC15	29.3
A16	OC16	29.9
A17	OC17	31.0
A21	OC21	28.8
A22	OC22	28.8
A23	OC23	33.2
A24	OC24	29.3
A25	OC25	29.3
A26	OC26	34.3
A28	OC28	27.7
A31	OC30	31.5
A32	OC32	27.2
A38	OC38	39.3
A39	OC39	27.7
A40	OC40	29.3
A41	OC41	29.9
A42	OC42	38.8
A43	OC43	31.5
A44	OC44	23.0
A45	OC45	30.4
	AVG	30.5

Table 5-1: TGFß and EGF levels in ascites fluid. A) Active and total (active + latent) TGFß was measured in 11 ascites samples. **B)** EGF levels were measured in 20 ascites samples.

A.

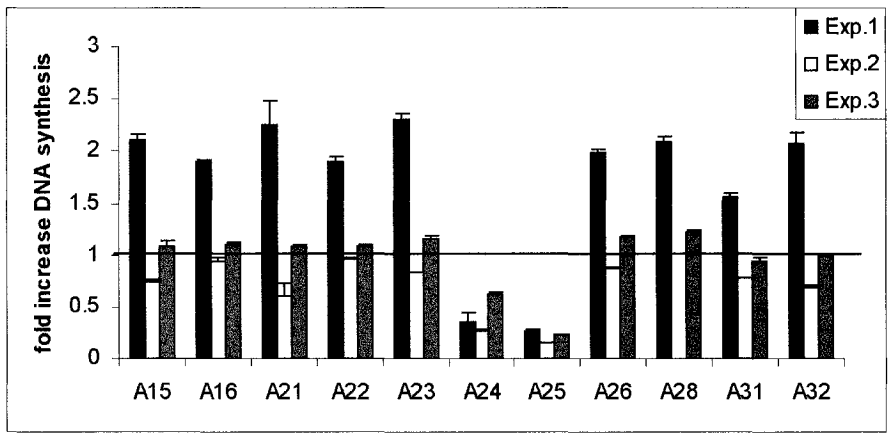
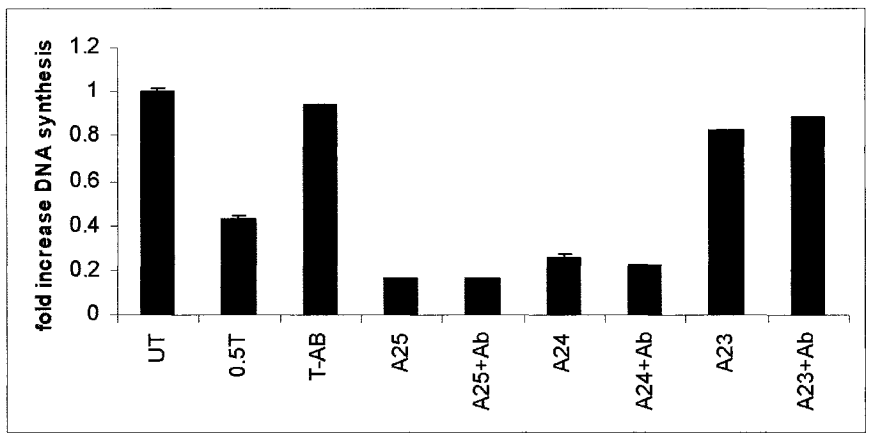


Figure 5-1. DNA synthesis of Mv1Lu cells in response to ascites fluid. Results shown are fold increase DNA synthesis compared to untreated (UT) Mv1Lu cells (set at 1). **A)** Inconsistent results were obtained in 3 experiments investigating DNA synthesis in response to ascites fluid. Experiment 1 (Exp.1) resulted in enhanced DNA synthesis, with the exception of A24 and A25. Experiment 2 (Exp.2) and experiment 3 (Exp.3) resulted in no change in DNA synthesis, again with A24 and A25 being exceptions. **B)** Neutralizing antibody against TGFß (Ab) did not reverse the inhibition of DNA synthesis in response to A24 and A25, although co-treatment with antibody (T-Ab) does reverse the inhibition of DNA synthesis seen with treatment with 0.5ng/ml TGFß (0.5T). **C)** The inhibition of DNA synthesis seen with 0.5ng/ml TGFß is inhibited by treatment with ascites fluid (A16). Increasing amounts of TGFß (1T and 5T) do not inhibit DNA synthesis when ascites fluid is present.

B.



C.

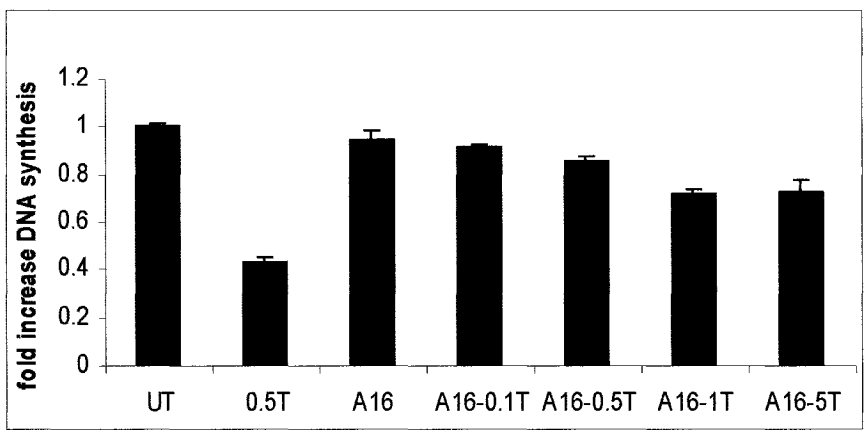


Figure 5-1. DNA synthesis of Mv1Lu cells in response to ascites fluid.

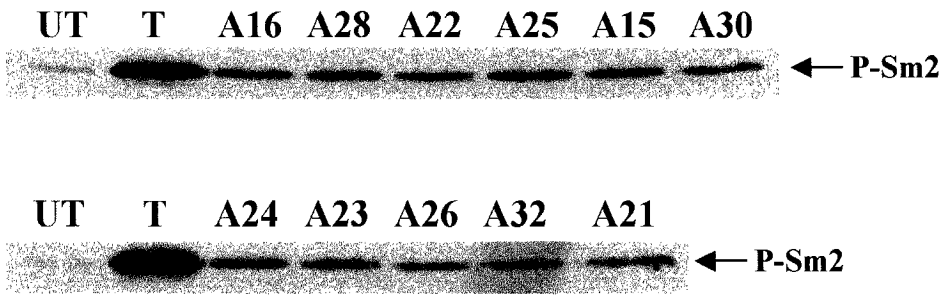
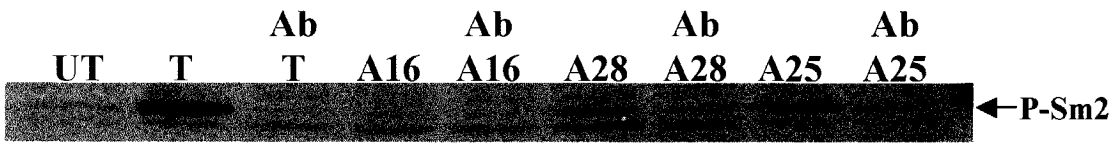


Figure 5-2: Phosphorylation of Smad2 in response to ascites fluid in SkOV3 cells. Western blot analysis of SkOV3 cells treated with ascites samples examining Smad2 phosphorylation (P-Sm2) levels. Untreated cells (UT) have little phosphorylated Smad2, which is strongly induced by treatment with 0.1ng/ml TGFß (T). Treatment with ascites (A) induces Smad2 phosphorylation.

A.



B.

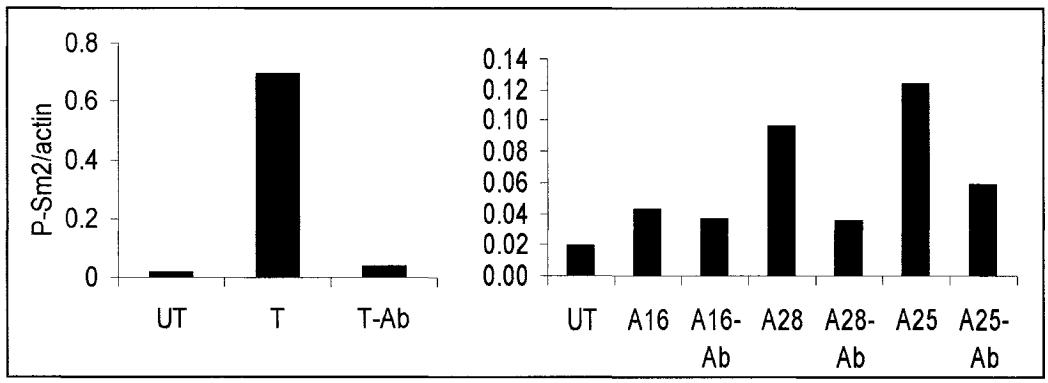


Figure 5-3: Phosphorylation of Smad2 in response to ascites fluid is blocked by TGFß neutralizing antibody. A) Untreated SkOV3 cells (UT) have little phosphorylated Smad2 protein (P-Sm2), and TGFß (T) treatment strongly induces phosphorylation of Smad2, which is completely blocked by co-treatment with TGFß neutralizing antibody (T-Ab). A slight increase in phosphorylated Smad2 is seen with ascites (A16, A28, A25) treatment, which is decreased by co-treatment with neutralizing antibody (Ab). **B)** Phospho-Smad2 (P-Sm2) levels normalized to ß-actin protein levels.

CHAPTER 6

SUMMARY, SIGNIFICANCE AND FUTURE STUDIES

6.1 SUMMARY

This study investigated the TGFß signaling pathway in primary ovarian cancer cells, to determine whether modifications in TGFß signaling may contribute to ovarian tumorigenesis. Although TGFß signaling defects are apparent in other types of cancer, such as colorectal, lung and pancreatic, no signaling defects were detected in ovarian cancer. Upon further investigation of the pathway, however, it can be suggested that other signaling pathways present *in vivo* may alter TGFß signaling, and contribute to uncontrolled proliferation of ovarian cancer cells.

TGFß1, TßRI, TßRII, Smad2, Smad3, Smad4 and SARA were found to be expressed in all primary ovarian cancer cell samples and ovarian cancer cell lines tested. Endogenous and transfected TGFß target genes were up-regulated in response to TGFß in primary ovarian cancer cells and cell lines, indicating the TGFß signaling pathway is functional. Moreover, primary ovarian cancer cells respond to the anti-proliferative effect of TGFß, which is likely mediated by up-regulation of the cell cycle regulator, $p15^{INK4B}$. In contrast, ovarian cancer cell lines and OC1 were found to be resistant to the anti-proliferative effect of TGFß. Ovarian cancer cell lines and OC1 have deleted the $p15^{INK4B}$ gene, and therefore, this deletion is likely the mechanism of resistance to the anti-proliferative effect of TGFß. Although the TGFß signaling pathway was found to be intact and functional in primary ovarian cancer cells, these cells continue to proliferate *in vivo*, and therefore, mechanisms must exist to inhibit TGFß signaling *in vivo*.

Other growth factor signaling pathways, such as the EGF pathway, have been shown to converge with the TGFß signaling pathway to modulate its effects. EGF receptor is expressed on OSE and ovarian cancer cells, and EGF is mitogenic to these

cells. Therefore, the effect of EGF signaling on the TGFß signaling pathway was tested. EGF inhibits the anti-proliferative effect of TGFß in primary ovarian cancer cells. Multiple steps within the TGFß pathway could be inhibited by EGF, and these possibilities were explored. EGF does not inhibit TGFß induced Smad phosphorylation or nuclear translocation, however, EGF does inhibit specific target gene expression. EGF had no effect on the TGFß-induced modulation of Smad7 or Id1, but did inhibit the TGFß-induced up-regulation of $p15^{INK4B}$. Therefore, the EGF inhibition of the anti-proliferative effect of TGFß is likely mediated by modulation of $p15^{INK4B}$ expression. A possible mechanism is via EGF stabilization of Smad transcriptional co-repressors, such as TGIF, which could inhibit TGFß-induced $p15^{INK4B}$ expression.

Although TGFß and EGF are likely present in ascites fluid of ovarian cancer patients, the levels were undetermined. Therefore, ELISAs were used to measure levels of active TGFß and EGF. TGFß was found to be present at high levels within ascites fluid, however, signaling activity of the TGFß was dampened. TGFß in ascites fluid was able to transmit a TGFß signal, however, the level of signaling was much lower than expected if all TGFß present is able to transmit a signal. Therefore, a significant portion of TGFß signaling must be inhibited prior to Smad activation, and the remaining intracellular signal may be inhibited by crosstalk from other factors, such as EGF. The levels of EGF, however, were found to be much lower than expected. Hence, other factors that signal in a manner similar to EGF, such as TGFα or oncogenic ras, could account for the inhibition of TGFß signaling.

Overall, this study demonstrates that primary ovarian cancer cells respond to the anti-proliferative effect of TGFß in cell culture, but this effect can be inhibited by EGF.

Although EGF levels are low in ascites fluid, other related factors may have a similar effect and contribute to uncontrolled cellular proliferation of ovarian cancer cells *in vivo* by altering the TGFß effect. Hence, although ovarian cancer cells in culture respond to the anti-proliferative effect of TGFß, the effect of TGFß *in vivo* is likely altered by additional signaling pathways resulting in ovarian cancer progression.

6.2 SIGNIFICANCE

Lack of early detection methods and effective therapies are factors that contribute to the high mortality rate of ovarian cancer. Understanding processes that lead to ovarian tumorigenesis and factors associated with disease progression are necessary to develop better detection methods and treatment options. This study investigates the role of TGFß in ovarian cancer, and processes that could alter TGFß signaling to enhance proliferation of ovarian cancer cells.

Considering TGFß signaling defects are implicated in other types of cancer, investigating this signaling pathway in ovarian cancer is a rational approach to better understand factors that contribute to ovarian cancer development. The contradictory reports in the literature examining TGFß signaling defects in ovarian cancer further provided incentive to investigate this pathway. Previous studies suggesting that mutations in components of the signaling pathway were responsible for resistance to the anti-proliferative effect of TGFß in other types of cancer did not correlate with this study. The TGFß signaling pathway is intact and functional in primary human ovarian cancer cells, which respond to the anti-proliferative effect of TGFß.

The bi-functional role that TGFß plays in tumor progression is of great importance to this study. Early in tumor development, TGFß acts as a tumor suppressor (Akhurst 2002; Tang et al. 1998), and cancer cells are sensitive to the anti-proliferative effect of TGFß. Later in tumor progression, however, TGFß switches from playing a role as a tumor suppressor, to acting as a tumor promoter by enhancing metastasis and angiogenesis (Akhurst 2002). Therefore, if the tumor promoting role of TGFß could be inhibited by therapeutic intervention, perhaps the tumor suppressor role would not be suppressed and cancer cells could be growth inhibited by TGFß.

Other factors produced by cancer cells likely cause this switch in the role of TGFß (Akhurst 2002). This study has shown that EGF signaling can inhibit the anti-proliferative effect of TGFß, and hence *in vivo*, factors such as EGF may cause ovarian cancer cells to be resistant to the anti-proliferative effect of TGFß and may also further contribute to tumorigenesis. TGFα, oncogenic ras, or HER2/neu would likely signal in a manner similar to EGF, and therefore, may contribute to inhibition of the anti-proliferative effect of TGFß *in vivo*.

Inhibition of the EGF signaling pathway may restore the anti-proliferative effect of TGFß, and be a potential therapeutic option for ovarian cancer patients. Targeted therapeutics are increasingly being developed and tested as cancer therapeutics. ZD1839 (Iressa; AstraZeneca) is an orally active selective EGFR tyrosine kinase inhibitor, that is currently being tested for use in other types of cancers, such as non small cell lung cancer, colorectal, breast and prostate cancer (Barker et al. 2001; Baselga and Averbuch 2000; Ciardiello 2000). ZD1839 does not affect other tyrosine kinase receptors, such as the receptors for VEGF or FGF and because ZD1839 does not completely block EGFR

activity, side effects for this drug are limited (Wakeling et al. 2002, Baselga and Averbuch 2000). In addition, oral bioavailability and half-life of this drug are good, making it a useful therapy (Barker et al. 2001, Baselga and Averbuch 2000). Perhaps inhibition of EGFR activity by ZD1839 or a similar drug could alter ovarian cancer cell responsiveness to TGFß, and be a useful therapy for ovarian cancer patients.

Due to the tumor promoting properties of TGFß, treatment with TGFß could have detrimental effects, and may not merely cause cancer cell growth arrest but may actually enhance tumor promotion. In fact, studies have suggested that inhibition of the TGFß signaling pathway using soluble TGFß type II receptors may have clinical applications, by inhibiting TGFß induced invasion and metastasis. Muraoka et al. (2002) and Yang et al. (2002) investigated the use of soluble TßRII, and found metastasis of breast cancer cells was inhibited in mice. Furthermore, it was suggested that although metastasis was inhibited, proliferation of the primary tumor was not affected, indicating that there was a specific effect on metastasis (Muraoka et al. 2002, Yang et al. 2002). TGFß inhibitors may be useful to prevent metastasis of cancer, however, inhibition of the tumor suppressive functions of TGFß must be avoided. Metastasis of ovarian cancer is very different from metastasis of breast cancer, and therefore, the same effects may not be seen.

The inhibition of the anti-proliferative effect of TGFß by EGF appears to be partially mediated by decreased $p15^{INK4B}$ expression. One proposed mechanism of inhibition is via TGIF stabilization by EGF (Figure 4-6). With the increased development of small molecule inhibitors as therapies for cancer, it would be rational to suggest that an inhibitor of a molecule within this proposed mechanism could be

inhibited, which may restore the TGFß induced up-regulation of $p15^{INK4B}$ and growth arrest. Inhibition of TGIF or HDAC may be feasible. HDAC inhibitors have been suggested to be potentially useful in cancer treatment (Yoshida et al. 2001), however, one problem is the general inhibition of a number of genes. Indeed, treatment of primary ovarian cancer cells with the HDAC inhibitor trichostatin A (TSA) results in cytotoxicity (L.D.D., data not shown). Therefore, treatment with a general HDAC inhibitor may not be a useful option, however, inhibition of TGIF as a therapeutic option remains possible.

These studies suggest a mechanism by which ovarian cancer cells may be resistant to the anti-proliferative effect of TGFß *in vivo*, and may provide insight into methods to overcome this resistance. Overall, better treatment strategies are required for ovarian cancer patients, and perhaps these studies will lead to clinical applications of current treatments such as ZD1839 or the development of other strategies to enhance ovarian cancer patient prognosis.

6.3 FUTURE STUDIES

6.3.1 The role of TGIF in EGF inhibition of the anti-proliferative effect of TGFß

One of the possible mechanisms by which EGF inhibits the anti-proliferative effect of TGFß is by stabilization of the Smad transcriptional co-repressor TGIF. In order to determine whether TGIF plays a role in this inhibition, studies investigating the interaction of TGIF and Smads at the $p15^{INK4B}$ promoter are necessary. Electromobility shift assays, DNaseI protection assays, and chromatin immunoprecipitation could be used to determine complex formation at the $p15^{INK4B}$ promoter in response to TGFß and EGF, alone and in combination. Supershifts using antibodies against TGIF and the Smads will

indicate whether these proteins are part of a complex. These studies will show whether EGF enhances formation of a TGIF/HDAC/Smad complex at the $p15^{INK4B}$ promoter, which would cause inhibition of $p15^{INK4B}$ expression.

6.3.2 ZD1839 treatment of primary ovarian cancer cells in combination with EGF and TGFß

Cell proliferation analysis of primary ovarian cancer cells treated with TGFß, EGF, and ZD1839, alone and in combination will determine whether specific inhibition of EGF signaling will recover the effects of TGFß. It is expected that treatment with TGFß, EGF and ZD1839 will result in growth arrest, due to the inhibition of the mitogenic effect of EGF and restoration of the anti-proliferative effect of TGFß.

6.3.3 EGFR and HER2/neu expression

RT-PCR analysis indicates EGFR is expressed in primary ovarian cancer cells (data not shown). Further analysis to examine expression of HER2/neu is also necessary, to determine whether expression of HER2/neu may play a role in inhibition of TGFß signaling. Overexpression of HER2/neu in primary ovarian cancer cells may result in ligand independent signaling, which may function in a manner similar to EGF signaling to inhibit the anti-proliferative effect of TGFß. Levels of EGFR and HER2/neu expression can be determined in primary ovarian cancer cells using Northern and Western analysis. Expression of these receptors in primary ovarian cancer cells will further suggest signaling via these receptors may act to alter TGFß signaling.

6.3.4 Mechanism of inhibition of ascitic TGFß

Although high levels of active TGFß were present in ascites fluid, minimal signaling activity was seen with the TGFß in ascites fluid. This study suggests that the

TGFß present in ascites fluid is inhibited prior to R-Smad phosphorylation, which may be at the level of ligand binding or receptor activation. The ELISA utilized TßRII to 'capture' active TGFß, thus it seems unlikely that TGFß is associated with an inhibitory protein preventing it from binding to TßRII. Hence, TGFß present in ascites fluid likely is able to bind TßRII. The possibility does exist however, that during the ELISA protocol, TGFß is released from inhibitory binding proteins and is able to bind TßRII. A possible TGFß binding protein that may inhibit TGFß binding to TßRII is fetuin. Fetuin is a serum component that shares homology with TßRII, and is able to bind TGFß1 (Demetriou et al. 1996). Studies have demonstrated inhibition of TGFß activity by fetuin, including inhibition of the anti-proliferative response (Demetriou et al. 1996). Hence, fetuin may be present in ascites fluid, thereby binding TGFß1 and inhibiting binding to TßRII and further signaling. In addition, α2-macroglobulin as been shown to bind TGFß resulting in inhibition (O'Connor-McCourt & Wakefield 1987), and hence, the presence of α2-macroglobulin in ascites fluid may act to inhibit TGFß signaling.

The other possibility by which signaling of ascitic TGFß is inhibited is by modulation of receptor activation. Kinase activity of TßRII or TßRI could be inhibited, which would prevent phosphorylation of TßRI or the R-Smads respectively. In vitro kinase assays to determine receptor activity could be conducted to determine if ascites fluid inhibits activity of either TßRI or TßRII. These future experiments would provide further insight into the mechanism of inhibition of the anti-proliferative effect of TGFß in primary ovarian cancer cells that may lead to novel therapeutics for the treatment of ovarian cancer.

REFERENCES

Abdollah S, Macias-Silva M, Tsukazaki T, Hayashi H, Attisano L, Wrana JL. 1997. TbetaRI phosphorylation of Smad2 on Ser465 and Ser467 is required for Smad2-Smad4 complex formation and signaling. J Biol Chem 272:27678-27685.

Abe M, Oda N, Sato Y. 1998. Cell-associated activation of latent transforming growth factor-ß by calpain. J Cell Physiol 174:186-193.

Abendstein B, Stadlmann S, Knabbe C, Buck M, Muller-Holzner E, Zeimet AG, Marth C, Obrist P, Krugmann J, Offner FA. 2000. Regulation of transforming growth factor-ß secretion by human peritoneal mesothelial and ovarian carcinoma cells. Cytokine. 12:1115-1119.

Abu-Jawdeh GM, Faix JD, Niloff J, Tognazzi K, Manseau E, Dvorak HF, Brown LF. 1996. Strong expression of vascular permeability factor (vascular endothelial growth factor) and its receptors in ovarian borderline and malignant neoplasms. Lab Invest 74:1105-1115.

Afrakhte M, Moren A, Jossan S, Itoh S, Sampath K, Westermark B, Heldin CH, Heldin NE, ten Dijke P. 1998. Induction of inhibitor smad6 and smad7 mRNA by TGF-ß family members. Biochem and Biophys Res Comm 249:505-511.

Akhurst RJ. 2002. TGF-ß antagonists: why suppress a tumor suppressor? J Clin Invest 109:1533-1536.

Akiyoshi S, Inoue H, Hanai J, Kusanagi K, Nemoto N, Miyazono K, Kawabata M. 1999. c-Ski acts as a transcriptional co-repressor in transforming growth factor-ß signaling through interaction with Smads. J Biol Chem 274:35269-35277.

Anderl P, Fuith LC, Daxenbichler G, Marth C, Dapunt O. 1988. Correlation between steroid hormone receptors, histological and clinical parameters in ovarian carcinoma. Gynecol Obstet Invest 25:135-140.

Anders RA, Arling SL, Doré JJE, Leof EB. 1997. Distinct endocytic responses of heteromeric and homomeric transforming growth factor ß receptors. Mol Biol Cell 8:2133-2143.

Ashcroft GS, Yang X, Glick AB, Weinstein M, Letterio JJ, Mizel DE, Anzano M, Greenwell-Wild T, Wahl SM, Deng C, Roberts AB. 1999. Mice lacking Smad3 show accelerated wound healing and an impaired local inflammatory response. Nature Cell Biol 1:260-266.

Assoian RK, Komoriya A, Meyers CA, Miller DM, Sporn MB. 1983. Transforming growth factor-beta in human platelets. Identification of a major storage site, purification, and characterization. J Biol Chem 258:7155-7160.

Attisano L, Wrana JL. 2002. Signal transduction by the TGF-ß superfamily. Science 296:1646-1647.

Auersperg N, Maines-Bandiera LS, Dyck HG, Kruk PA. 1994. Characterization of cultured human ovarian surface epithelial cells: phenotypic plasticity and premalignant changes. Lab Invest 71:510-518.

Auersperg N, Edelson MI, Mok SC, Johnson SW, Hamilton TC. 1998. The biology of ovarian cancer. Sem Oncol 25:281-304.

Auersperg N, Pan J, Grove BD, Peterson T, Fisher J, Maines-Bandiera S, Somasiri A, Roskelley CD. 1999. E-cadherin induces mesenchymal-to-epithelial transition in human ovarian surface epithelium. Proc Natl Acad Sci 96:6249-6254.

Auersperg N, Wong AST, Choi K-C, Kang SK, Leung PCK. 2001. Ovarian surface epithelium: biology, endocrinology, and pathology. Endocrine Rev 22:255-288.

Barbara NP, Wrana JL, Letarte M. 1999. Endoglin is an accessory protein that interacts with the signaling receptor complex of multiple members of the transforming growth factor-ß superfamily. J Biol Chem 274:584-594.

Barker AJ, Gibson KH, Grundy W, Godfrey AA, Barlow JJ, Healy MP, Woodburn JR, Ashton SE, Curry BJ, Scarlett L, Henthorn L, Richards L. 2001. Studies leading to the identification of ZD1839 (Iressa): an orally active, selective epidermal growth factor receptor tyrosine kinase inhibitor targeted to the treatment of cancer. Bioorg Med Chem Lett 11:1911-1914.

Baselga J, Averbuch SD. 2000. ZD1839 ('Iressa') as an anticancer agent. Drugs 60 Suppl:33-40.

Bast RC Jr, Feeney M, Lazarus H, Nadler LM, Colvin RB, Knapp RC. 1981. Reactivity of a monoclonal antibody with human ovarian carcinoma. Clin Invest 68:1331-1337.

Bast RC Jr, Klug TL, St.John E, Jenison E, Niloff JM, Lazarus H, Berkowitz RS, Leavitt T, Griffiths CT, Parker L, Zurawski VR, Knapp RC. 1983. A radioimmunoassay using a monoclonal antibody to monitor the course of epithelial ovarian cancer. N Engl J Med 309:883-887.

Battaglia F, Scambia G, Benedetti Panici P, Baiocchi G, Perrone L, Iacobelli S, Mancuso S. 1989. Epidermal growth factor receptor expression in gynecological malignancies. Gynecol Obstet Invest 27:42-44.

Beck EP, Russo P, Gliozzo B, Jaeger W, Papa V, Wildt L, Pezzino V, Lang N. 1994. Identification of insulin and insulin-like growth factor 1 (IGF1) receptors in ovarian cancer tissue. Gynecol Oncol 53:196-201.

Berchuck A, Rodriguez GC, Kamel A, Dodge RK, Soper JT, Clark-Pearson DL, Bast RC. 1991. Epidermal growth factor receptor expression in normal and ovarian epithelium and ovarian cancer. I: Correlation of receptor expression with prognosis factors in patients with ovarian cancer. Am J Obstet Gynecol 164:669-674.

Berchuck A, Rodriguez G, Olt G, Whitaker R, Boente MP, Arrick BA, Clarke-Pearson DL, Bast RC. 1992. Regulation of growth of normal ovarian epithelial cells and ovarian cancer cell lines by transforming growth factor-ß. Am J Obstet Gynecol 166:676-684.

Berchuck A, Kohler MF, Boente MP, Rodriguez GC, Whitaker RS, Bast RC. 1993. Growth regulation and transformation of ovarian epithelium. Cancer. 71:545-551.

Berns EMJJ, Klijn JGM, Henzen-Logmans SC, Rodenburg CJ, van der Burg MEL, Joekens JA. 1992. Receptors for hormones and growth factors and (onco)-gene amplifications in human ovarian cancer. Int J Cancer 52:218-224.

Blaustein A, Kaganawicz A, Wells J. 1982. Tumor markers in inclusion cysts of the ovary. Cancer 49:722-726.

Bonni S, Wang HR, Causing CG, Kavsak P, Stroschein SL, Lui K, Wrana JL. 2001. TGF-ß induces assembly of a Smad2-Smurf2 ubiquitin ligase complex that targets SnoN for degradation. Nature Cell Biol 3:587-595.

Bosetti C, Negri E, Trichopoulos D, Franceschi S, Beral V, Tzonou A, Parazzini F, Greggi S, La Vecchia C. 2002. Long-term effects of oral contraceptives on ovarian cancer risk. Int J Cancer 102:262-265.

Boyd FT, Massague J. 1989. Growth inhibitory response to transforming growth factor-ß linked to expression of a 53 kDa cell surface TGF-ß receptor. J Biol Chem 264:2272-2278.

Bristow RE, Baldwin RL, Yamada SD, Korc M, Karlan BY. 1999. Altered expression of transforming growth factor-ß ligands and receptors in primary and recurrent ovarian carcinoma. Cancer 85:658-668.

Burke F, Relf M, Negus R, Balkwill F. 1996. A cytokine profile of normal and malignant ovary. Cytokine 8:578-585.

Carcamo J, Zentella A, Massague J. 1995. Disruption of transforming growth factor beta signaling by a mutation that prevents transphosphorylation within the receptor complex. Mol Cell Biol 15:1573-1581.

Charng M-J, Zhang D, Kinnunen P, Schneider MD. 1998. A novel protein distinguishes between quiescent and activated forms of the type I transforming growth factor ß receptor. J Biol Chem 273:9365-9368.

Cheifetz S, Weatherbee JA, Tsang MLS, Anderson JK, Mole JE, Lucas R, Massague J. 1987. The transforming growth factor-ß system, a complex pattern of cross-reactive ligands and receptors. Cell 48:409-415.

Cheifetz S, Bellon T, Cales C, Vera S, Bernabeu C, Massague J, Letarte M. 1992. Endoglin is a component of the transforming growth factor-beta receptor system in human endothelial cells. J Biol Chem 267:19027-19030.

Chen RH, Miettinen PJ, Maruoka EM, Choy L, Derynck R. 1995. A WD-domain protein that is associated with and phosphorylated by the type II TGF-ß receptor. Nature 377:548-552.

Chen T, Carter D, Garrigue-Antar L, Reiss M. 1998. Transforming growth factor ß type I receptor kinase mutant associated with metastatic breast cancer. Cancer Res 58:4805-4810.

Chen T, de Vries EGE, Hollema H, Yegen HA, Vellucci VF, Strickler HD, Hildesheim A, Reiss M. 1999. Structural alterations of transforming growth factor-ß receptor genes in human cervical carcinoma. Int J Cancer 82:43-51.

Chen T, Triplett J, Dehner B, Hurst B, Colligan B, Pemberton J, Graff JR, Carter JH. 2001. Transforming growth factor-ß receptor type I gene is frequently mutated in ovarian carcinoma. Cancer Res 61:4679-4682.

Chen YG, Liu F, Massague J. 1997. Mechanism of TGFß receptor inhibition by FKBP12. EMBO J 16:3866-3876.

Chien C-H, Huang C-C, Lui Y-H, Shen J, Chow S-N. 1997. Detection of serum transforming growth factor-α in patients of primary epithelial ovarian cancers by enzyme immunoassay. Gynecol Oncol 66:405-410.

Chipuk JE, Cornelius SC, Pultz NJ, Jorgensen JS, Bonham MJ, Kim SJ, Danielpour D. 2002. The androgen receptor represses transforming growth factor-ß signaling through interaction with Smad3. J Biol Chem 277:1240-1248.

Choi KC, Kang SK, Tai CJ, Auersperg N, Leung PCK. 2001. The regulation of apoptosis by activin and transforming growth factor-ß in early neoplastic and tumorigenic ovarian surface epithelium. J Clin Endo Met 86:2125-2135.

Chomczynski P, Sacchi N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156-159.

Choy L, Derynck R. 1998. The type II transforming growth factor (TGF)-ß receptor-interacting protein TRIP-1 acts as a modulator of the TGF-ß response. J Biol Chem 273:31455-31462.

Ciardiello F. 2000. Epidermal growth factor receptor tyrosine kinase inhibitors as anticancer agents. Drugs 60 Suppl:25-32.

Coffey RJ, Bascom CC, Sipes NJ, Graves-Deal R, Weissman BE, Moses HL. 1988. Selective inhibition of growth-related gene expression in murine keratinocytes by transforming growth factor ß. Mol Cell Biol 8:3088-3093.

Connelly DC, Bao R, Nikitin AY, Stephens KC, Poole TW, Hua X, Harris SS, Vanderhyden BC, Hamilton TC. 2003. Female mice chimeric for expression of the simian virus 40 TAg under control of the MISIIR promoter develop epithelial ovarian cancer. Cancer Res 63:1389-1397.

Conover CA, Hartman LC, Bradley S, Stalboerger P, Klee GG, Kalli KR, Jenkins RB. 1998. Biological characterization of human epithelial ovarian carcinoma cells in primary culture: the insulin-like growth factor system. Exp Cell Res 238:439-449.

Corps AN, Sowter HM, Smith SK. 1997. Hepatocyte growth factor stimulates motility, chemotaxis and mitogenesis in ovarian carcinoma cells expressing high levels of c-MET. Int J Cancer 73:151-155.

Crawford SE, Stellmach V, Murphy-Ullrich JE, Ribeiro SMF, Lawler J, Hynes RO, Boivin GP, Bouck N. 1998. Thrombospondin-1 is a major activator of TGF-ß1 in vivo. Cell 93:1159-1170.

Crew AJ, Langdon SP, Miller EP, Miller WR. 1992. Mitogenic effects of epidermal growth factor and transforming growth factor-α on EGF-receptor positive human ovarian carcinoma cell lines. Eur J Cancer 28:337-341.

Crickard K, Gross JL, Crickard U, Yoonessi M, Lele S, Herblin WF, Eidsvoog K. 1994. Basic fibroblast growth factor and receptor expression in human ovarian cancer. Gynecol Oncol 55:277-284.

Dabrow MB, Francesco MR, McBreaty FX, Caradonna S. 1998. The effects of platelet-derived growth factor and receptor on normal and neoplastic human ovarian surface epithelium. Gynecol Oncol 71:29-37.

Dang CV. 1999. C-Myc targets genes involved in cell growth , apoptosis, and metabolism. Mol Cell Biol 19:1-11.

Datta PK, Chytil A, Gorska AE, Moses HL. 1998. Identification of STRAP, a novel WD domain protein in transforming growth factor-ß signaling. J Biol Chem 273:34671-34674.

Datta PK, Moses HL. 2000. STRAP and Smad7 synergize in the inhibition of transforming growth factor ß signaling. Mol Cell Biol 20:3157-3167.

Datta PK, Blake MC, Moses HL. 2000. Regulation of plasminogen activator inhibitor-1 expression by transforming growth factor-ß-induced physical and functional interactions between Smads and Sp1. J Biol Chem 275:40014-40019.

Datto MB, Li Y, Panus JF, Howe DJ, Xiong Y, Wang XF. 1995a. Transforming growth factor ß induced the cyclin-dependent kinase inhibitor p21 through a p53-independent mechanism. Proc Natl Acad Sci 92:5545-5549.

Datto MB, Yu Y, Wang XF. 1995b. Functional analysis of the transforming growth factor ß responsive elements in the WAF1/Cip1/p21 promoter. J Biol Chem 270:28623-28628.

Datto MB, Frederick JP, Pan L, Borton AJ, Zhuang Y, Wang XF. 1999. Targeted disruption of Smad3 reveals an essential role in transforming growth factor beta-mediated signal transduction. Mol Cell Biol 19:2495-2504.

Davis HM, Zurawski VR, Bast RC Jr, Klug TL. 1986. Characterization of the CA 125 antigen associated with human epithelial ovarian carcinomas. Cancer Res 46:6143-6148.

De Caestecker MP, Parks WT, Frank CJ, Castagnino P, Bottaro DP, Roberts AB, Lechleider RJ. 1998. Smad2 transduces common signals from receptor serine-threonine and tyrosine kinases. Genes Dev 12:1587-1592.

DeCoteau JF, Knaus PI, Yankelev H, Reis MD, Lowsky R, Lodish HF, Kadin ME. 1997. Loss of functional cell surface transforming growth factor ß (TGF-ß) type I receptor correlates with insensitivity to TGF-ß in chronic lymphocytic leukemia. Proc Natl Acad Sci 94:5877-5881.

Demetriou M, Binkert C, Sukhu B, Tenenbaum HC, Dennis JW. 1996. Fetuin/α2-HS glycoprotein is a transforming growth factor-ß type II receptor mimic and cytokine antagonist. J Biol Chem 271:12755-12761.

Dennler S, Itoh S, Vivien D, ten Dijke P, Huet S, Gauthier JM. 1998. Direct binding of Smad3 and Smad4 to critical TGF beta-inducible elements in the promoter of human plasminogen activator inhibitor-type I gene. EMBO J 17:3091-3100.

Derynck R, Jarrett JA, Chen EY, Eaton DH, Bell JR, Assoian RK, Roberts AB, Sporn MB, Goeddel DV. 1985. Human transforming growth factor-ß complementary DNA sequence and expression in normal and transformed cells. Nature 316:701-705.

Di Guglielmo GM, Le Roy C, Goodfellow AF, Wrana JF. 2003. Distinct endocytic pathways regulate TGF-ß receptor signaling and turnover. Nature Cell Biol 5:410-421.

Di Renzo MF, Olivero M, Katsaros D, Crepaldi T, Gaglia P, Zola P, Sismondi P, Comoglio PM. 1994. Overexpression of the MET/HGF receptor in ovarian cancer. Int J Cancer 58:658-662.

Diebold RJ, Eis MJ, Yin M, Ormsby I, Boivin GP, Darrow BJ, Saffitz JE, Doetschman T. 1995. Early-onset multifocal inflammation in the transforming growth factor beta 1-null mouse is lymphocyte mediated. Proc Natl Acad Sci 92:12215-12219.

Doré JJE, Yao D, Edends M, Garamazegi N, Sholl EL, Leof EB. 2001. Mechanisms of transforming growth factor-ß receptor endocytosis and intracellular sorting differ between fibroblasts and epithelial cells. Mol Biol Cell 12:675-684.

Du Bois A. 2001. Treatment of advanced ovarian cancer. Eur J Cancer 37:S1-S7.

Dubois CM, Laprise M-H, Blanchette F, Gentry LE, Leduc R. 1995. Processing of transforming growth factor ß1 precurser by human furin convertase. J Biol Chem 270:10618-10624.

Dunfield LD, Campbell Dwyer EJ, Nachtigal MW. 2002. TGFß-induced Smad signaling remains intact in primary human ovarian cancer cells. Endocrinology 143:1174-1181.

Dunfield LD, Shepherd TG, Nachtigal MW. 2002. Primary culture and mRNA analysis of human ovarian cells. Biological Procedures Online 4:55-61.

Dunfield LD, Nachtigal MW. 2003. Inhibition of the anti-proliferative effect of TGFß by EGF in primary human ovarian cancer cells. Oncogene. *In press*.

Elbendary A, Berchuck A, Davis P, Havrilesky L, Bast RC, Iglehart D, Marks JR. 1994. Transforming growth factor ß1 can induce CIP1/WAF1 expression independent of the p53 pathway in ovarian cancer cells. Cell Growth Diff 5:1301-1307.

Emons G, Ortmann O, Becker M, Irmer G, Springer B, Laun R, Holzel F, Schulz K-D, Schally AV. 1993. High affinity binding and direct antiproliferative effects of LHRH analogues in human ovarian cancer cell lines. Cancer Res 53:5439-5446.

Enomoto T, Inoue M, Perantoni AO, Terakawa N, Tanizawa O, Rice JM. 1990. K-ras activation in neoplasms of the human female reproductive tract. Cancer Res 50:6139-6145.

Enomoto T, Weghorst CM, Inoue M, Tanizawa O, Rice JM. 1991. K-ras activation occurs frequently in mucinous adenocarcinomas and rarely in other common epithelial tumors of the human ovary. Am J Pathol 139:777-785.

Eppert K, Scherer SW, Ozcelik H, Pirone R, Hoodless P, Kim H, Tsui LC, Bapat B, Gallinger S, Andrulis IL, Thomsen GH, Wrana JL, Attisano L. 1996. MADR2 maps to 18q21 and encodes a TGFß-regulated MAD-related protein that is functionally mutated in colorectal cancer. Cell 86:543-552.

Evangelou A, Jindal SK, Brown TJ, Letarte M. 2000. Down-regulation of transforming growth factor ß receptors by androgen in ovarian cancer cells. Cancer Res 60:929-935.

Facchini LM and Penn LZ. 1998. The molecular role of myc in growth and transformation: recent discoveries lead to new insights. FASEB J 12:633-651.

Fathalla MF. 1971. Incessant ovulation – a factor in ovarian neoplasia? Lancet 2:163.

Feng XH, Zhang Y, Wu RY, Derynck R. 1998. The tumor suppressor Smad4/DPC4 and transcriptional adaptor CBP/p300 are coactivators for smad3 in TGF-beta-induced transcriptional activation. Genes Dev 12:2153-2163.

Feng XH, Lin X, Derynck R. 2000. Smad2, Smad3 and Smad4 cooperate with Sp1 to induce $p15^{Ink4B}$ transcription in response to TGF-ß. EMBO J 19:5178-5193.

Feng XH, Liang YY, Liang M, Zhai W, Lin X. 2002. Direct interaction of c-myc with Smad2 and Smad3 to inhibit TGF-ß-mediated induction of the CDK inhibitor $p15^{Ink4B}$. Mol Cell 9:133-143.

Ford LC, Berek JS, Lagasse LD, Hacker NF, Heins Y, Esmailian F, Leuchter RS, DeLange RJ. 1983. Estrogen and progesterone receptors in ovarian neoplasms. Gynecol Oncol 15:299-304.

Francis-Thickpenny KM, Richardson DM, van Ee CC, Love DR, Winship IM, Baguley BC, Chenevix-Trench C, Shelling AN. 2001. Analysis of the TGFß functional pathway in epithelial ovarian carcinoma. Br J Cancer 85:687-691.

Fukuchi M, Imamura T, Chiba T, Ebisawa T, Kawabata M, Tanaka K, Miyazono K. 2001. Ligand-dependent degradation of Smad3 by a ubiquitin ligase complex of ROC1 and associated proteins. Mol Biol Cell 12:1431-1443.

Funaba M, Zimmerman CM, Mathews LS. 2002. Modulation of Smad2-mediated signaling by extracellular signal-regulated kinase. J Biol Chem 277:41361-41368.

Garrett AP, Lee KR, Colitti CR, Muto MG, Berkowitz RS, Mok SC. 2001. K-ras mutation may be an early event in mucinous ovarian tumorigenesis. Int J Gynecol Pathol 20:244-251.

Gibbs DD, Gore ME. 2001. Pursuit of optimum outcomes in ovarian cancer: methodological approaches to therapy. Drugs 61:1103-1119.

Gold LI. 1999. The role for transforming growth factor-ß (TGF-ß) in human cancer. Crit Rev Oncog 10:303-360.

Gougos A, Letarte M. 1990. Primary structure of endoglin, an RGD-containing glycoprotein of human endothelial cells. J Biol Chem 265:8361-8364.

Graycar JL, Miller DA, Arrick BA, Lyons RM, Moses HL, Derynck R. 1989. Human transforming growth factor-beta 3: recombinant expression, purification, and biological activities in comparison with transforming growth factors-beta 1 and –beta 2. Mol Endocrinol 3:1977-1986.

Grimaldi G, Di Fiore P, Locatelli EK, Falco J, Blasi F. 1986. Modulation of urokinase plasminogen activator gene expression during the transition from quiescent to proliferative state in normal mouse cells. EMBO J 5:855-861.

Hahn SA, Schutte M, Hoque ATMS, Moskaluk CA, da Costa LT, Rozenblum E, Weinstein CL, Fischer A, Yeo CJ, Hruban RH, Kern SE. 1996. DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1. Science 271:350-353.

Haldane JS, Hird V, Hughes CM, Gullick WJ. 1990. *c-erbB-2* oncogene expression in ovarian cancer. J Pathol 162:231-237.

Hankinson SE, Colditz GA, Hunter DJ, Willett WC, Stampfer MJ, Rosner B, Hennekens CH, Speizer FE. 1995. A prospective study of reproductive factors and risk of epithelial ovarian cancer. Cancer 76:284-290.

Hannon GJ, Beach D. 1994. $p15^{INK4B}$ is a potential effector of TGF-ß-induced cell cycle arrest. Nature 371:257-261.

Harding M, Cowan S, Hole D, Cassidy L, Kitchener H, Davis J, Leake R. 1990. Estrogen and progesterone receptors in ovarian cancer. Cancer 65:486-491.

Hata A, Lo RS, Wotton D, Lagna G, Massague J. 1997. Mutations increasing autoinhibition inactivate tumour suppressors Smad2 and Smad4. Nature 388:82-87.

Havrilesky LJ, Hurteau JA, Whitaker RS, Elbendary A, Wu S, Rodriquez GC, Bast RC, Berchuck A. 1995. Regulation of apoptosis in normal and malignant ovarian epithelial cells by transforming growth factor ß. Cancer Res 55:944-948.

Hayashi H, Abdollah S, Qiu Y, Cai J, Xu Y-Y, Grinnell BW, Richardson MA, Topper JN, Gimbrone MA, Wrana JL, Falb D. 1997. The MAD-related protein Smad7 associates with the TGFß receptor and functions as an antagonist of TGFß signaling. Cell 89:1165-11173.

Heldin CH, Miyazono K, ten Dijke P. 1997. TGF-ß signalling from cell membrane to nucleus through SMAD proteins. Nature 390:465-471.

Hempling RE. 1994. Tumor markers in epithelial ovarian cancer: clinical applications. In: Gershenson DM, ed. Update on epithelial ovarian cancer. Philadelphia: WB Saunders Co.; vol 21:41-61.

Henriksen R, Funa K, Wilander E, Backstrom T, Ridderheim M, Oberg K. 1993. Expression and prognostic significance of platelet-derived growth factor and its receptors in epithelial ovarian neoplasms. Cancer Res 53:4550-4554.

Henriksen R, Gobl A, Wilander E, Oberg K, Miyazono K, Funa K. 1995. Expression and prognostic significance of TGF-ß isotypes, latent TGF-ß1 binding protein, TGF-ß type I and type II receptors, and endoglin in normal ovary and ovarian neoplasms. Lab Invest 73:213-220.

Hirabayashi K, Graham J. 1970. Genesis of ascites in ovarian cancer. Am J Obstet Gynecol 106:492-497.

Hirte HW, Clarke DA, Mazurka J, O'Connell G, Rusthoven J. 1992. A rapid and simple method for the purification of tumor cells from ascitic fluid of ovarian carcinoma. Gynecol Oncol 44:223-226.

Hoskins WJ. 1993. Surgical staging and cytoreductive surgery of epithelial ovarian cancer. Cancer 71:1534-1540.

Hua X, Lui X, Ansari DO, Lodish HF. 1998. Synergistic cooperation of TFE3 and smad proteins in the TGF-ß-induced transcription of the plasminogen activator inhibitor-1 gene. Genes Dev 12:3084-3095.

Hua X, Miller ZA, Benchabane H, Wrana JL, Lodish HF. 2000. Synergism between transcription factors TFE3 and Smad3 in transforming growth factor-ß-induced transcription of the Smad7 gene. J Biol Chem 275:33205-33208.

Hurteau J, Rodriguez GC, Whitaker RS, Shah S, Mills G, Bast RC, Berchuck A. 1994. Transforming growth factor-beta inhibits proliferation of human ovarian cancer cells obtained from ascites. Cancer 74:93-99.

Ibanez-Tallon I, Ferrai C, Longobardi E, Facetti I, Blasi F, Crippa MP. 2002. Binding of Sp1 to the proximal promoter links constitutive expression of the human uPA gene and invasive potential of PC3 cells. Blood 100:3325-3332.

Imoto I, Pimkhaokham A, Watanabe T, Saito-Ohara F, Soeda E, Inazawa J. 2000. Amplification and Overexpression of TGIF2, a novel homeobox gene of the TALE superclass, in ovarian cancer cell lines. Biochem Biophys Res Commun 276:264-270.

Inman GJ, Hill CS. 2002. Stoichiometry of active Smad-transcription factor complexes on DNA. J Biol Chem 277:51008-51016.

Inman GJ, Nicolas FJ, Hill CS. 2002. Nucleocytoplasmic shuttling of Smads 2, 3, and 4 permits sensing of TGF-ß receptor activity. Mol Cell 10:283-294.

International Federation of Gynecology and Obstetrics. 1971. Classification and staging of malignant tumours in the female pelvis. Acta Obstet Gynec Scand 50:1-7.

Itoh S, Landstrom M, Hermansson A, Itoh F, Heldin CH, Heldin NE, ten Dijke P. 1998. Transforming growth factor ß1 induces nuclear export of inhibitory Smad7. J Biol Chem 273:29195-29201.

Iverson OE, Skaarland E, Utaaker E. 1986. Steroid receptor content in human ovarian tumors: survival of patients with ovarian carcinoma related to steroid receptor content. Gynecol Oncol 23:65-76.

Janda E, Lehmann K, Killisch I, Jechlinger M, Herzig M, Downward J, Beug H, Grunert S. 2002. Ras and TGFß cooperatively regulate epithelial cell plasticity and metastasis: dissection of Ras signaling pathways. J Cell Biol 156:299-313.

Janknecht R, Wells NJ, Hunter T. 1998. TGF-beta-stimulated cooperation of smad proteins with the coactivators CBP/p300. Genes Dev 12:2114-2119.

Kaartinen V, Voncken JW, Shuler C, Warburton D, Bu D, Heisterkamp N, Groffen J. 1995. Abnormal lung development and cleft palate in mice lacking TGF-beta3 indicates defects of epithelial-mesenchymal interaction. Nat Genet 11:415-421.

Kabawat S, Bast RC, Welch WR, Knapp RC, Colvin RB. 1983. Immunopathologic characterization of a monoclonal antibody that recognizes common surface antigens of human ovarian tumors of serous, endometrioid, and clear cell types. Am J Clin Pathol 79:98-104.

Kang SH, Bang YJ, Im YH, Yang HK, Lee DA, Lee HW, Lee HS, Kim NK, Kim SJ. 1999. Transcriptional repression of the transforming growth factor-ß type I receptor gene by DNA methylation results in the development of TGF-ß resistance in human gastric cancer. Oncogene 18:7280-7286.

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

Karlan BY, Jones J, Greenwald M, Lagasse LD. 1995. Steroid hormone effects on the proliferation of human ovarian surface epithelium in vitro. Am J Obstet Gynecol 173:97-104.

Kavsak P, Rasmussen RK, Causing CG, Bonni S, Zhu H, Thomsen GH, Wrana JL. 2000. Smad7 binds to Smurf2 to form an E3 ubiquitin ligase that targets the TGFß receptor for degradation. Mol Cell 6:1365-1375.

Kehrl JH, Roberts AB, Wakefield LM, Jakowlew S, Sporn MB, Fauci AS. 1986. Transforming growth factor ß is an important immunomodulatory protein for human B lymphocytes. J Immunol 137:3855-3860.

Keski-Oja J, Raghow R, Sawdey M, Loskutoff DJ, Postlethwaite AE, Kang AH, Moses HL. 1988a. Regulation of mRNA for type-1 plasminogen activator inhibitor, fibronectin, and type I procollagen by transforming growth factor-beat. Divergent responses in lung fibroblasts and carcinoma cells. J Biol Chem 263:3111-3115.

Keski-Oja J, Blasi F, Leof EB, Moses HL. 1988b. Regulation of the synthesis and activity of urokinase plasminogen activator in A549 human lung carcinoma cells by transforming growth factor-ß. J Cell Biol 106:451-459.

Kim IY, Ahn HJ, Zelner DJ, Shaw JW, Sensibar JA, Kim JH, Kato M, Lee C. 1996. Genetic change in transforming growth factor ß (TGF-ß) receptor type I gene correlates with insensitivity to TGF-ß1 in human prostate cancer cells. Cancer Res 56:44-48.

Kim J, Johnson K, Chen HJ, Carroll S, Laughon A. 1997. Drosophila Mad binds to DNA and directly mediates activation of vestigial by Decapentaplegic. Nature 388:304-308.

Kitazume H, Kitamura K, Mukai K, Inayama Y, Kawano N, Nakamura N, Sano J, Mitsui K,, Yoshida S, Nakatani Y. 2000. Cytologic differential diagnosis among reactive mesothelial cells, malignant mesothelioma, and adenocarcinoma. Cancer Cytopathol 90:55-60.

Kohler M, Bauknecht T, Grimm M, Birmelin G, Kommoss F, Wagner E. 1992. Epidermal growth factor receptor and transforming growth factor alpha expression in human ovarian carcinomas. Eur J Cancer 28:1432-1437.

Kretzschmar M, Doody J, Timokhina I, Massague J. 1999. A mechanism of repression of TGFß/Smad signaling by oncogenic ras. Genes Dev 13:804-816.

Kulkarni AB, Huh CG, Becker D, Geiser A, Lyght M, Flanders KC, Roberts AB, Sporn MB, Ward JM, Karisson S. 1993. Transforming growth factor beta 1 null mutation in mice causes excessive inflammatory response and early death. Proc Natl Acad Sci 90:770-774.

Kurasaki A, Kose S, Yoneda Y, Heldin CH, Moustakas A. 2001. Transforming growth factor-ß induces nuclear import of Smad3 in an importin-ß1 and ran-dependent manner. Mol Biol Cell 12:1079-1091.

Labbé E, Silvestri C, Hoodless PA, Wrana JL, Attisano L. 1998. Smad2 and Smad3 positively and negatively regulate TGF beta-dependent transcription through the forkhead DNA-binding protein FAST2. Mol Cell 2:109-120.

Lagna G, Hata A, Hemmati-Brivanlou A, Massague J. 1996. Partnership between DPC4 and SMAD proteins in TGF-beta signaling pathways. Nature 383:832-836.

Lastres P, Letamendia A, Zhang H, Rius C, Almendro N, Raab U, Lopez LA, Langa C, Fabra A, Letarte M, Bernabeu C. 1996. Endoglin modulates cellular responses to TGF-ß1. J Cell Biol 133:1109-1121.

Li CY, Suardet L, Little JB. 1995. Potential role of WAF1/Cip1/p21 as a mediator of TGF-ß cytoinhibitory effect. J Biol Chem 270:4971-4974.

Li JM, Nichols MA, Chandrasekharan S, Xiong Y, Wang XF. 1995. Transforming growth factor ß activates the promoter of cyclin-dependent kinase inhibitor $p15^{INK4B}$ though an Sp1 consensus site. J Biol Chem 270:26750-26753.

Lin HY, Wang XF, Ng-Eaton E, Weinberg RA, Lodish HF. 1992. Expression cloning of the TGF-beta type II receptor, a functional transmembrane serine/threonine kinase. Cell 68:775-785.

Lin X, Liang M, Feng XH. 2000. Smurf2 is a ubiquitin E3 ligase mediating proteosome-dependent degradation of Smad2 in transforming growth factor-ß signaling. J Biol Chem 275:36818-36822.

Lo RS, Chen YG, Shi Y, Pavletich NP, Massague J. 1998. The L3 loop: a structural motif determining specific interactions between SMAD proteins and TGF-beta receptors. EMBO J 17:996-1005.

Lo RS, Massagué J. 1999. Ubiquitin-dependent degradation of TGF-ß-activated Smad2. Nature Cell Biol 1:472-478.

Lo RS, Wotton D, Massague J. 2001. Epidermal growth factor signaling via Ras controls the Smad transcriptional co-repressor TGIF. EMBO J 20:128-136.

Lopez-Casillas F, Wrana JL, Massague J. 1993. Betaglycan presents ligand to the TGF beta signaling receptor. Cell 73:1435-1444.

Lu Z, Murray JT, Luo W, Li H, Wu X, Xu H, Backer JM, Chen Y-G. 2002. Transforming growth factor ß activates Smad2 in the absence of receptor endocytosis. J Biol Chem 277:29363-29368.

Lund LR, Riccio A, Andreasen PA, Nielsen LS, Kristensen P, Laiho M, Saksela O, Blasi F, Dano K. 1987. Transforming growth factor-ß is a strong and fast acting positive regulator of the level of type-1 plasminogen activator inhibitor mRNA in WI-38 human lung fibroblasts. EMBO J 6:1281-1286

Luo K, Stroschein SL, Wang W, Chen D, Martens E, Zhou S, Zhou Q. 1999. The Ski oncoprotein interacts with the Smad proteins to repress TGFß signaling. Genes Dev 13:2196-2206.

Lynch MA, Nakashima R, Song H, DeGroff VL, Wang D, Enomoto T, Weghorst CM. 1998. Mutational analysis of the transforming growth factor ß receptor type II gene in human ovarian carcinoma. Cancer Res 58:4227-4232.

Lyons RM, Gentry LE, Purchio AF, Moses HL. 1990. Mechanism of activation of latent recombinant transforming growth factor ß1 by plasmin. J Cell Biol. 110:1361-1367.

Macias-Silva M, Abdollah S, Hoodless PA, Pirone R, Attisano L, Wrana JL. 1996. MADR2 is a substrate of the TGFbeta receptor and its phosphorylation is required for nuclear accumulation and signaling. Cell 87:1215-1224.

Maines-Bandiera SL, Auersperg N. 1997. Increased E-cadherin expression in ovarian surface epithelium: an early step in metaplasia and dysplasia. Int J Gynecol Pathol 16:250-255.

Malik S, Balkwill F. 1991. Epithelial ovarian cancer: a cytokine propelled disease? Br J Cancer 64:617-620.

Mandai M, Konishi I, Kuroda H, Fukumoto M, Komatsu T, Yamamoto S, Nanbu K, Rao CV, Mori T. 1997. Messenger ribonucleic acid expression of LH/hCG receptor gene in human ovarian carcinomas. Eur J Cancer 33:1501-1507.

Markowitz S, Wang J, Myeroff L, Parsons R, Sun L, Lutterbaugh J, Fan RS, Zborowska E, Kinzler KW, Vogelstein B, Brattain M, Willson JKV. 1995. Inactivation of the type II TGF-ß receptor in colon cancer cells with microsatellite instability. Science 268:1336-1277.

Marth C, Zeimet AG, Herold M, Brumm C, Windbichler G, Muller-Holzner E, Offner F, Feichtinger H, Zwierzina H, Daxenbichler G. 1996. Different effects of interferons, interleukin-1ß and tumor necrosis factor-α in normal OSE and malignant human ovarian epithelial cells. Int J Cancer 67:826-830.

Massagué J. 1992. Receptors of the TGF-ß family. Cell 69:1067-1070.

Massagué J. 1998. TGF-ß signal transduction. Annu Rev Biochem 67:753-791.

Massagué J, Wotton D. 2000. Transcriptional control by the TGF-ß/Smad signaling system. EMBO J 19:1745-1754.

Masui T, Wakefield LM, Lechner JF, LaVeck MA, Sporn MB, Harris CC. 1986. Type ß transforming growth factor is the primary differentiation-inducing serum factor for normal human bronchial epithelial cells. Proc Natl Acad Sci 83:2438-2442.

Matsuda T, Yamamoto T, Muraguchi A, Saatcioglu F. 2001. Cross-talk between transforming growth factor-ß and estrogen receptor signaling through Smad3. J Biol Chem 276:42908-42914.

McAllister KA, Grogg KM, Johnson DW, Gallione CJ, Baldwin MA, Jackson CE, Helmbold EA, Markel DS, McKinnon WC, Murrell J, McCormick MK, Pericak-Vance MA, Heutink P, Oostra BA, Haitjema T, Westerman CJJ, Porteous ME, Guttmacher AE, Letarte M, Marchuck DA. 1994. Endoglin, a TGF-beta binding protein of endothelial cells, is the gene for hereditary haemorrhagic telangiectasia. Nat Genet 8:345-351.

Meersseman G, Verschueren K, Nelles L, Blumenstock C, Kraft H, Wuytens G, Remacle J, Kozak CA, Tylzanowski P, Niehrs C, Huylebroeck D. 1997. The C-terminal domain of Mad-like signal transducers is sufficient for biological activity in the Xenopus embryo and transcriptional activation. Mech Dev 61:127-140.

Melhuish TA, Gallo CM, Wotton D. 2001. TGIF2 interacts with histone deacetylase I and represses transcription. J Biol Chem 276:32109-32114.

Merwin JR, Newman W, Beall LD, Tucker A, Madri J. 1991. Vascular cells respond differently to transforming growth factors beta 1 and beta 2 in vitro. Am J Pathol 138:37-51.

Miyazono K, Hellman U, Wernstedt C, Heldin CH. 1988. Latent high molecular weight complex of transforming growth factor beta 1. Purification from human platelets and structural characterization. J Biol Chem 263:6401-6415.

Miyazono K, Olofsson A, Colosetti P, Heldin CH. 1991. A role of the latent TGF-ß1-binding protein in the assembly and secretion of TGF-ß1. EMBO J 10:1091-1101.

Morishige K, Kurachi H, Amemiya K, Fujita Y, Yamamoto T, Miyake A, Tanizawa O. 1991. Evidence for the involvement of transforming growth factor α and epidermal growth factor receptor autocrine growth mechanism in primary human ovarian cancers *in vitro*. Cancer Res 51:5322-5328.

Muraoka RS, Dumont N, Ritter CA, Dugger TC, Brantley DM, Chen J, Easterly E, Roebuck LR, Ryan S, Gotwals PJ, Koteliansky V, Arteaga CL. 2002. Blockade of TGF-beta inhibits mammary tumor cell viability, migration, and metastasis. J Clin Invest 109:1551-1559.

Myeroff LL, Parsons R, Kim SJ, Hedrick L, Cho KR, Orth K, Mathis M, Kinzler KW, Lutterbaugh J, Park K, Bang YJ, Lee HY, Park JG, Lynch HT, Roberts AB, Vogelstein B, Markowitz SD. 1995. A transforming growth factor ß type II gene mutation common in colon and gastric but rare in endometrial cancers with microsatellite instability. Cancer Res 55:5545-5547.

Nagatake M, Takagi Y, Osada H, Uchida K, Mitsudomi T, Saji S, Shimokata K, Takahashi T, Takahashi T. 1996. Somatic in vivo alteration of the DPC4 gene at 18q21 in human lung cancers. Cancer Res 56:2718-2720.

Nakao A, Imamura T, Souchelnytskyi S, Kawabata M, Ishisaki A, Oeda E, Tamaki K, Hanai J, Heldin CH, Miyazono K, ten Dijke P. 1997a. TGF-beta receptor-mediated signaling through Smad2, Smad3 and Smad4. EMBO J 16:5353-5362.

Nakao A, Afrakhte M, Moren A, Nakayama T, Christian JL, Heuchel R, Itoh S, Kawabata M, Heldin NE, Heldin CH, ten Dijke P. 1997b. Identification of Smad7, a TGFß-inducible antagonist of TGF-ß signaling. Nature 389:631-635.

Nerlov C, Rorth P, Blasi F, Johnsen M. 1991. Essential AP-1 and PEA3 binding elements in the human urokinase enhancer display cell type-specific activity. Oncogene 6:1583-1592.

Nerlov C, de Cesare D, Pergola F, Caracciola A, Blasi F, Johnsen M, Verde P. 1992. A regulatory element that mediates co-operation between a PEA2-AP1 element and an AP-1 site is required for phorbol ester induction of urokinase enhancer activity in HepG2 hepatoma cells. EMBO J 11:4573-4582.

Niedbala MJ and Sartorelli AC. 1989. Regulation by epidermal growth factor of human squamous cell carcinoma plasminogen activator-mediated proteolysis of extracellular matrix. Cancer Res 49:3302-3309.

Nunes I, Gleizes PE, Metz CN, Rifkin DB. 1997. Latent transforming growth factor-beta binding protein domains involved in activation and transglutaminase-dependent cross-linking of latent transforming growth factor-beta. J Cell Biol 136:1151-1163.

O'Connor-McCourt M, Wakefield LM. 1987. Latent transforming growth factor-ß in serum. A specific complex wsith α2-macroglobulin. J Biol Chem 262:14090-14099.

Oft M, Peli J, Rudaz C, Schwarz H, Beug H, Reichmann E. 1996. TGF-ß1 and Ha-Ras collaborate in modulating the phenotype plasticity and invasiveness of epithelial tumor cells. Genes Dev 10:2462-2477.

Oft M, Heider KH, Beug H. 1998. TGFß signaling is necessary for carcinoma cell invasiveness and metastasis. Curr Biol 8:1243-1252.

Ogata T, Wozney JM, Benezra R, Noda M. 1993. Bone morphogenetic protein 2 transiently enhances expression of a gene, Id (inhibitor of differentiation), encoding a helix-loop-helix molecule in osteoblast-like cells. Proc Natl Acad Sci 90:9219-9222.

Ong A, Maines-Bandiera SL, Roskelley CD, Auersperg N. 2000. An ovarian adenocarcinoma line derived from SV40/E-cadherin-transfected normal human ovarian surface epithelium. Int J Cancer 85:430-437.

Onichtchouk D, Chen Y-G, Dosch R, Gawantka V, Delius H, Massague J, Niehrs C. 1999. Silencing of TGF-ß signaling by the pseudoreceptor BAMBI. Nature 401:480-485.

Osada H, Tatematsu Y, Masuda A, Saito T, Sugiyama M, Yanagisawa K, Takahashi T. 2001. Heterogeneous transforming growth factor (TGF)-ß unresponsiveness and loss of TGF-ß receptor type II expression caused by histone deacetylation in lung cancer cell lines. 2001. Cancer Res 61:8331-8339.

Osterholzer HO, Streibel EJ, Nicosia SV. 1985. Growth effects of protein hormones on cultured rabbit ovarian surface epithelial cells. Biol Reprod 33:247-258.

Owens OJ, Steward C, Leake RE. 1991a. Growth factors in ovarian cancer. Br J Cancer 64:177-1181.

Owens OJ, Stewart C, Brown I, Leake RE. 1991b. Epidermal growth factor receptors (EGFR) in human ovarian cancer. Br J Cancer 64:907-910.

Ozols RF. 2002. Future directions in the treatment of ovarian cancer. Sem Oncol 29:32-42.

Park K, Kim SJ, Bang YJ, Park JG, Kim NK, Roberts AB, Sporn MB. 1994. Genetic changes in the transforming growth factor ß (TGF-ß) type II receptor gene in human gastric cancer cells: correlation with sensitivity to growth inhibition by TGF-ß. Proc Natl Acad Sci 91:8772-8776.

Peirreux CE, Nicolas FJ, Hill CS. 2000. Transforming growth factor ß-independent shuttling of Smad4 between the cytoplasm and nucleus. Mol Cell Biol 20:9041-9054.

Penheiter SG, Mitchell H, Garamszegi N, Edens M, Doré JJE, Leof EB. 2002. Internalization-dependent and –independent requirements for transforming growth factor ß receptor signaling via the smad pathway. Mol Cell Biol 22:4750-4759.

Petricoin EF, Ardekani AM, Hitt BA, Levine PJ, Fusaro VA, Steinberg SM, Mills GB, Simone C, Fishman DA, Kohn EC, Liotta LA. 2002. Use of proteomic patterns in serum to identify ovarian cancer. Lancet 359:572-577.

Piek E, Heldin C-H, Ten Dijke P. 1999. Specificity, diversity, and regulation in TGF-ß superfamily signaling. FASEB J 13:2105-2124.

Pietenpol JA, Holt JT, Stein RW, Moses HL. 1990. Transforming growth factor ß1 suppression of c-myc gene transcription: role in inhibition of keratinocyte proliferation. Proc Natl Acad Sci 87:3758-3762.

Polyak K, Lee MH, Erdjument-Bromage H, Koff A, Roberts JM, Tempst P, Massague J. 1994a. Cloning of $p27^{Kip1}$, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. Cell 78:59-66.

Polyak K, Kato JY, Solomon MJ, Sherr CJ, Massague J, Roberts JM, Koff A. 1994b. p27Kip1, a cyclin-Cdk inhibitor, links transforming growth factor-beta and contact inhibition to cell cycle arrest. Genes Dev 8:9-22.

Pouponnot C, Jayaraman L, Massague J. 1998. Physical and functional interaction of SMADs and p300/CBP. J Biol Chem 273:22865-22868.

Proetzel G, Pawlowski SA, Wiles MV, Yin M, Boivin GP, Howles PN, Ding J, Ferguson MW, Doetschman T. 1995. Transforming growth factor-beta 3 is required for secondary palate fusion. Nat Genet 11:40-414.

Prunier C, Mazars A, Noe V, Bruyneel E, Mareel M, Gespach C, Atfi A. 1999. Evidence that Smad2 is a tumor suppressor implicated in the control of cellular invasion. J Biol Chem 274:22919-22922.

Punnonen R, Teisala K, Kuoppala T, Bennett B, Punnonen J. 1998. Cytokine production profiles in the peritoneal fluids of patients with malignant or benign gynecologic tumors. Cancer 83:788-796.

Resta L, Russo S, Colucci GA, Prat J. 1993. Morphologic precursors of ovarian epithelial tumors. Obstet Gynecol 82:181-186.

Reynisdottir I, Polyak K, Iavarone A, Massague J. 1995. Kip/Cip and Ink4 Cdk inhibitors cooperate to induce cell cycle arrest in response to TGF-ß. Genes Dev 9:1831-1845.

Riggins GJ, Kinzler KW, Vogelstein B, Thiagalingam S. 1997. Frequency of Smad gene mutations in human cancer. Cancer Res 57:2578-2580.

Risch HA, Weiss NS, Lyon JL, Daling JR, Liff JM. 1983. Events of reproductive life and the incidence of epithelial ovarian cancer. Am J Epidemiol 117:128-139.

Roberts AB, Anzano MA, Lamb LC, Smith JM, Sporn MB. 1981. New class of transforming growth factors potentiated by epidermal growth factor: isolation from non-neoplastic tissues. Proc Natl Acad Sci 78:5339-5343.

Roberts AB, Sporn MB, Assoian RK, Smith FM, Roche NS, Wakefield LM, Heine UI, Liotta LA, Falanga V, Kehrl JH, Fauci AS. 1986. Transforming growth factor type ß: rapid induction of fibrosis and angiogenesis in vivo and stimulation of collagen formation in vitro. Proc Natl Acad Sci 83:4167-4171.

Roberts AB, Sporn MB. 1992. Differential expression of the TGF-ß isoforms in embryogenesis suggests specific roles in developing and adult tissues. Mol Reprod Dev 32:91-98.

Rodriguez C, Chen F, Weinberg RA, Lodish HF. 1995. Cooperative binding of transforming growth factor (TGF)-beta 2 to the types I and II TGF-beta receptors. J Biol Chem 270:15919-15922.

Rodriguez GC, Berchuck A, Whitaker RS, Schlossman D, Clarke-Pearson DL, Bast RC. 1991. Epidermal growth factor receptor expression in normal ovarian epithelium and ovarian cancer. II: Relationship between receptor expression and response to epidermal growth factor. Am J Obstet Gynecol 164:745-750.

Rodriquez GC, Walmer DK, Cline M, Krigman H, Lessey BA, Whitaker RS, Dodge R, Hughes CL. 1998. Effect of progestin on the ovarian epithelium of macaques: cancer prevention through apoptosis? J Soc Gynecol Investig 5:271-276.

Rodriguez GC, Nagarsheth NP, Lee KL, Bentley RC, Walmer DK, Cline M, Whitaker RS, Isner P, Berchuck A, Dodge RK, Hughes CL. 2002. Progestin-induced apoptosis in the macaque ovarian epithelium: differential regulation of transforming growth factor-ß. J Natl Cancer Inst 94:50-60.

Rosenberg L, Shapiro S, Slone D, Kaufman DW, Helmrich SP, Miettinen OS, Stolley PD, Rosenshein NB, Schottenfeld D, Engle RL. 1982. Epithelial ovarian cancer and combination oral contraceptives. JAMA 247:3210-3212.

Rosenberg L, Palmer JR, Zauber AG, Warshauer ME, Lewis JL, Strom BL, Harlap S, Shapiro S. 1994. A case-control study of oral contraceptive use and invasive epithelial ovarian cancer. Am J Epidemiol 139:654-661.

Royar J, Becher H, Chang-Claude J. 2001. Low-dose oral contraceptives: protective effect on ovarian cancer risk. Ink J Cancer 95:370-374.

Saltzman AK, Hartenback EM, Carter JR, Contreras DN, Twiggs LB, Carson LF, Ramakrishnan S. 1999. Transforming growth factor-alpha levels in the serum and ascites of patients with advanced epithelial ovarian cancer. Gynecol Obstet Invest 47:200-204.

Sanford LP, Ormsby I, Gittenberger-de Groot AC, Sariola H, Friedman R, Boivin GP, Cardell EL, Doetschman T. 1997. TGFbeta2 knockout mice have multiple developmental defects that are non-overlapping with other TGFbeta knockout phenotypes. Development 124:2659-2670.

Sano Y, Harada J, Tashiro S, Gotoh-Mandeville R, Mackawa T, Ishii S. 1999. ATF-2 is a common nuclear target of Smad and TAK1 pathways in transforming growth factor-beta signaling. J Biol Chem 274:8949-8957.

Schildkraut JM, Calingaert B, Marchbanks PA, Moorman PG, Rodriguez GC. 2002. Impact of progestin and estrogen potency in oral contraceptives on ovarian cancer risk. J Natl Cancer Inst 94:32-38.
Schofield K, D'Aquila T, Rimm DL. 1997. The cell adhesion molecule, E-cadherin, distinguishes mesothelial cells from carcinoma cells in fluids. Cancer (Cancer Cytopathol) 81:293-293.

Schultz-Cherry S, Murphy-Ullrich JE. 1993. Thrombospondin causes activation of latent transforming growth factor-ß secreted by endothelial cells by a novel mechanism. J Cell Biol 122:923-932.

Schutte M, Hruban RH, Hedrick L, Cho KR, Nadasdy GM, Weinstein CL, Bova GS, Isaacs WB, Cairns P, Nawroz H, Sidransky D, Casero RA, Meltzer PS, Hahn SA, Kern SE. 1996. DPC4 gene in various tumor types. Cancer Res 56:2527-2530.

Schwarz LC, Gingras MC, Goldberg G, Greenberg AH, Wright JA. 1988. Loss of growth factor dependence and conversion of transforming growth factor-ß1 inhibition to stimulation in metastatic H-ras-transformed murine fibroblasts. Cancer Res 48:6999-7003.

Scully RE. 1995. Pathology of ovarian cancer precursor. J Cell Biochem Suppl 23:208-218.

Senger DR, Galli SJ, Dvorak AM, Perruzzi CA, Harvey VS, Dvorak HF. 1983. Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. Science 219:983-985.

Seoane J, Pouponnot C, Staller P, Schader M, Eilers M, Massague J. 2001. TGFß influences myc, miz-1 and smad to control the CDK inhibitor $p15^{INK5b}$. Nature Cell Biol 3:400-408.

Shen X, Hu PP, Liberati NT, Datto MB, Frederick JP, Wang XF. 1998. TGF-beta-induced phosphorylation of Smad3 regulates its interaction with coactivator p300/CREB-binding protein. Mol Biol Cell 9:3309-3319.

Sherr CJ. 1994. G1 phase progression: cycling on cue. Cell 79:551-555.

Shi Y, Wang YF, Jayaraman L, Yang H, Massague J, Pavletich NP. 1998. Crystal structure of a smad MH1 domain bound to DNA: insights on DNA binding in TGF-beta signaling. Cell 94:585-594.

Shull MM, Ormsby I, Kier AB, Pawlowski S, Diebold RJ, Yin M, Allen R, Sidman C, Proetzel G, Calvin D, Annunziata N, Doetschman T. 1992. Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease. Nature 359:693-699.

Siemens CH, Auersperg N. 1988. Serial propagation of human ovarian surface epithelium in tissue culture. J Cell Physiol 134:347-356.
Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE, Levin WJ, Stuart SG, Udove J, Ullrich A, Press MF. 1989. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. Science 244:707-712.

Somanna A, Mundodi V, Gedamu L. 2002. Functional analysis of cathepsin B-like cysteine proteases from *Leishmania donovani* complex: evidence for the activation of latent transforming growth factor ß. J Biol Chem 277:25305-25312.

Souchelnytskyi S, ten Dijke P, Miyazono K, Heldin CH. 1996. Phosphorylation of Ser165 in TGF-beta type I receptor modulates TGF-beta1-induced cellular responses. EMBO J 15:6231-6240.

Souchelnytskyi S, Tamaki K, Engstrom U, Wernstedt C, ten Dijke P, Heldin CH. 1997. Phosphorylation of Ser475 and Ser467 in the C terminus of Smad2 mediates interaction with Smad4 and is required for transforming growth factor-beta signaling. J Biol Chem 272:28107-28115.

Sowter HM, Corps AN, Smith SK. 1999. Hepatocyte growth factor (HGF) in ovarian epithelial tumor fluids stimulates the migration of ovarian carcinoma cells. Int J Cancer 83:476-480.

Staller P, Peukert K, Kiermaier A, Seoane J, Lukas J, Karsunky H, Moroy T, Bartek J, Massague J, Hanel F, Ellers M. 2001. Repression of p15INK4b expression by Myc through association with Miz-1. Nature Cell Biol 3:392-399.

Stratton JA, DiSaia PJ. 1981. Malignant ascites associated with advanced gynecologic neoplastic disease. Am J Obstet Gynecol 141:843-845.

Straube RL. 1958. Fluid accumulation during initial stages of ascites tumor growth. Cancer Res 18:57-65.

Stromberg K, Collins TJ, Gordon AW, Jackson CL, Johnson GR. 1992. Transforming growth factor-α acts as an autocrine growth factor in ovarian carcinoma cell lines. Cancer Res 52:341-347.

Stroschein SL, Wang W, Zhou S, Zhou Q, Luo K. 1999. Negative feedback regulation of TGFß signaling by the SnoN oncoprotein. Science 286:771-774.

Sundfeldt K, Piontkewitz Y, Ivarsson K, Nilsson O, Hellberg P, Brannstrom M, Janson PO, Enerback S, Hedin L. 1997. E-cadherin expression in human epithelial ovarian cancer and normal ovary. Int J Cancer 74:275-289.

Sun Y, Liu X, Eaton EN, Lane WS, Lodish HF, Weinberg RA. 1999. Interaction of the ski oncoprotein with Smad3 regulates TGF-ß signaling. Mol Cell 4:499-509.

Taipale J, Miyazono K, Heldin CH, Keski-Oja J. 1994. Latent transforming growth factor-beta 1 associates to fibroblast extracellular matrix via latent TGF-beta binding protein. J Cell Biol 124:171-181.

Takagi Y, Kohmura H, Futamura M, Kida H, Tanemura H, Shimokawa K, Saji S. 1996. Somatic alterations of the DPC4 gene in human colorectal cancers in vivo. Gastroenterology 111:1369-1372.

Tang B, Bottinger EP, Jakowlew SB, Bagnall KM, Mariano J, Anver MR, Letterio JJ, Wakefield LM. 1998. Transforming growth factor-ß1 is a new form of tumor suppressor with true haploid insufficiency. Nature Med 4:802-807.

ten Dijke P, Miyazono K, Heldin CH. 2000. Signaling inputs converge on nuclear effectors in TGF-ß signaling. TIBS 25:64-70.

Thiagalingam S, Lengauer C, Leach FS, Schutte M, Hahn SA, Overhauser J, Willson JKV, Markowitz S, Hamilton SR, Kern SE, Kinzler KW, Vogelstein B. 1996. Evaluation of candidate tumour suppressor genes on chromosome 18 in colorectal cancers. Nature Genetics 13: 343-346.

Tortolero-Luna G, Mitchell MF, Rhodes-Morris HE. 1994. Epidemiology and screening of ovarian cancer. In: Gershenson DM, ed. Update on epithelial ovarian cancer. Philadelphia: WB Saunders Co.; vol 21:1-23.

Tsukazaki T, Chiang TA, Davison AF, Attisano L, Wrana JL. 1998. SARA, a FYVE domain protein that recruits Smad2 to the TGFß receptor. Cell 95:779-791.

Uchida K, Nagatake M, Osada H, Yatabe Y, Kondo M, Mitsudomi T, Masuda A, Takahashi T, Takahashi T. 1994. Somatic in vivo alterations of the JV18-1 gene at 18q21 in human lung cancers. Cancer Res 56:5583-5585.

Ueoka Y, Kato K, Kuriaki Y, Horiuchi S, Terao Y, Nishida J, Ueno H, Wake N. 2000. Hepatocyte growth factor modulates motility and invasiveness of ovarian carcinomas via Ras-mediated pathway. Br J Cancer 82:891-899.

Ulloa L, Doody J, Massague J. 1999. Inhibition of transforming growth factor-ß/Smad signaling by the interferon-γ/STAT pathway. Nature 397:710-713.

Vigne JL, Halburnt LL, Skinner MK. 1994. Characterization of bovine ovarian surface epithelium and stromal cells: identification of secreted proteins. Biol Reprod 51:1213-1221.

von Gersdorff G, Susztak K, Rezvani F, Bitzer M, Liang D, Bottinger EP. 2000. Smad3 and Smad4 mediate transcriptional activation of the human Smad7 promoter by transforming growth factor ß. J Biol Chem 275:11320-11326.

Wakefield LM, Smith DM, Flanders KC, Sporn MB. 1988. Latent transforming growth factor-beta from platelets. A high molecular weight complex containing precursor sequences. J Biol Chem 263:7646-7654.

Wakefield LM, Winokur TS, Hollands RS, Christopherson K, Levinson AD, Sporn MB. 1990. Recombinant latent transforming growth factor ß1 has a longer plasma half-life in rats than active transforming growth factor ß1, and a different tissue distribution. J Clin Invest 86:1976-1984.

Wakeling AE, Guy SP, Woodburn JR, Ashton SE, Curry BJ, Barker AJ, Gibson KH. 2002. ZD1839 (Iressa): an orally active inhibitor of epidermal growth factor signaling with potential for cancer therapy. Cancer Res 62:5749-5754.

Wan M, Cao X, Wu Y, Bai S, Wu L, Shi X, Wang N, Cao. 2002. Jab1 antagonizes TGF-beta signaling by inducing Smad4 degradation. EMBO Rep 3:171-176.

Wang D, Kanuma T, Mizumuna H, Takama F, Ibuki Y, Wake N, Mogi A, Shitara Y, Takenoshita S. 2000. Analysis of specific gene mutations in the transforming growth factor-ß signal transduction pathway in human ovarian cancer. Cancer Res 60:4507-4512.

Wang T, Li B-Y, Danielson PD, Shah PC, Rockwell S, Lechleider RJ, Martin J, Manganaro T, Donohoe PK. 1996a. The immunophilin FKBP12 functions as a common inhibitor of the TGFß family type I receptors. Cell 86:435-444.

Wang T, Danielson PD, Li B, Shah PC, Kim SD, Donahoe PK. 1996b. The $p21^{RAS}$ farnesyltransferase α subunit in TGF-ß and activin signaling. Science. 271:1120-1122.

Warner BJ, Blain SW, Seoane J, Massague J. 1999. Myc downregulation by transforming growth factor ß required for activation of the $p15^{Ink4b}$ G1 arrest pathway. Mol Cell Biol 19:5913-5922.

Watson JE, Gabra H, Taylor KJ, Rabiasz GJ, Morrison H, Perry P, Smyth JF, Porteous DJ. 1999. Identification and characterization of a homozygous deletion found in ovarian ascites by representational difference analysis. Genome Res 9:226-233.

Weiss NS, Lyon JL, Liff JM, Vollmer WM, Daling JR. 1981. Incidence of ovarian cancer in relation to the use of oral contraceptives. Int J Cancer 28:669-671

Whittemore AS, Harris R, Itnyre J, Collaborative Ovarian Cancer Group. 1992. Characteristics relating to ovarian cancer risk: collaborative analysis of 12 US case-

control studies. II. Invasive epithelial ovarian cancers in white women. Am J Epidemiol 136:1184-1203.

Westerhausen DR, Hopkins WE, Billadello JJ. 1991. Multiple transforming growth factor-ß-inducible elements regulate expression of the plasminogen activator inhibitor type-1 gene in Hep G2 cells. J Biol Chem 266:1092-1100.

Wong AST, Maines-Bandiera SL, Rosen B, Wheelock WJ, Johnson KR, Leung PCK, Roskelley CD, Auersperg N. 1999. Constitutive and conditional cadherin expression in cultured human ovarian surface epithelium: influence of family history of ovarian cancer. Int J Cancer 81:180-188.

Wong C, Rougier-Chapman EM, Frederick JP, Datto MB, Liberati NT, Li JM, Wang XF. 1999. Smad3-Smad4 and AP-1 complexes synergize in transcriptional activation of the c-Jun promoter by transforming growth factor beta. Mol Cell Biol 19:1821-1830.

Wotton D, Lo RS, Lee S, Massague J. 1999a. A smad transcriptional corepressor. Cell 97:29-39.

Wotton D, Lo RS, Swaby LC, Massague J. 1999b. Multiple modes of repression by the smad transcriptional corepressor TGIF. J Biol Chem 274:37105-37110.

Wotton D, Knoepfler PS, Laherty CD, Eisenman RN, Massague J. 2001. The smad transcriptional corepressor TGIF recruits mSin3. Cell Growth Diff 12:457-463.

Wrana JL, Attisano L, Wiser R, Ventura F, Massague J. 1994. Mechanism of activation of the TGF-ß receptor. Nature 370:341-347.

Wu RYE, Zhang Y, Feng XH, Derrick R. 1997. Heteromeric and Homomeric interactions correlate with signaling activity and functional cooperatively of Smad3 and Smad4/DPC4. Mol Cell Biol 17:2521-2528.

Xiao Z, Luis X, Hens YI, Lodish HF. 2000a. A distinct nuclear localization signal in the N terminus of Smad3 determines its ligand-induced nuclear translocation. Proc Natl Acad Sci 97:7853-7858.

Xiao Z, Luis X, Lodish HF. 2000b. Importin ß mediates nuclear translocation of Smad 3. J Biol Chem 275:23425-23428.

Xu J, Attisano L. 2000. Mutations in the tumor suppressors Smad2 and Smad4 inactivate transforming growth factor ß signaling by targeting Smads to the ubiquitin-proteosome pathway. Proc Natl Acad Sci 97:4820-4825.

Xu L, Chen Y-G, Massague J. 2000. The nuclear import function of Smad2 is masked by SARA and unmasked by TGFß-dependent phosphorylation. Nature Cell Biol 2:559-562.

Xu L, Kang Y, Coi S, Massague J. 2002. Smad2 nucleocytoplasmic shuttling by nucleoporins CAN/Nup214 and Nup153 feeds TGFß signaling complexes in the cytoplasm and nucleus. Mol Cell 10:271-282.

Yamada SD, Baldwin RL, Karlan BY. 1999. Ovarian carcinoma cell cultures are resistant to TGF-ß1-mediated growth inhibition despite expression of functional receptors. Gynecol Oncol 75:72-77.

Yamao F. 1999. Ubiquitin system: selectivity and timing of protein destruction. J Biochem 125:223-229.

Yamashita H, Ichijo H, Grimsby S, Moren A, ten Dijke P, Miyazono K. 1994. Endoglin forms a heteromeric complex with the signaling receptors for transforming growth factor-beta. J Biol Chem 269:1995-2001.

Yang X, Letterio JJ, Lechleider RJ, Chen L, Hayman R, Gu H, Roberts AB, Deng C. 1999. Targeted disruption of SMAD3 results in impaired mucosal immunity and diminished T cell responsiveness to TGF-ß. EMBO J 18:1280-1291.

Yang YA, Dukhanina O, Tang B, Mamura M, Letterio JJ, MacGregor J, Patel SC, Khozin S, Liu ZY, Green J, Anver MR, Merlino G, Wakefield LM. 2002. Lifetime exposure to a soluble TGF-beta antagonist protects mice against metastasis without adverse effects. J Clin Invest 109:1607-1615.

Yee D, Morales FR, Hamilton TC, Von Hoff DD. 1991. Expression of insulin-like growth factor I, its binding properties, and its receptor in ovarian cancer. Cancer Res 51:5107-5112.

Yoshida M, Furumai R, Nishiyama M, Komatsu Y, Nishino N, Horinouchi S. 2001. Histone deacetylase as a new target for cancer chemotherapy. Cancer Chemother Pharmacol 48:S20-S26.

Yu Q, Stamenkovic I. 2000. Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-ß and promotes tumor invasion and angiogenesis. Genes Dev 14:163-176.

Zawel L, Dai JL, Buckhaults P, Zhou S, Kinzler KW, Vogelstein B, Kern SE. 1998. Human Smad3 and Smad4 are sequence-specific transcriptional activators. Mol Cell 1:611-617.

Zeinoun Z, Teugels E, de Bleser PJ, Neyns B, Geerts A, de Greve J. 1999. Insufficient TGF-ß1 production inactivates the autocrine growth suppressive circuit in human ovarian cancer cell lines. Anticancer Res 19:413-420.

Zentella A, Weis FMB, Ralph DA, Laiho M, Massague J. 1991. Early gene responses to transforming growth factor-ß in cells lacking growth-suppressive RB function. Mol Cell Biol 11:4952-4958.

Zhang Y, Feng X, We R, Derynck R. 1996. Receptor-associated Mad homologues synergize as effectors of the TGF-beta response. Nature 383:168-172.

Zhang Y, Feng XH, Derynck R. 1998. Smad3 and smad4 cooperate with c-Jun/c-Fos to mediate TGF-ß-induced transcription. Nature 394:909-913.

Zhang Y, Chang C, Gehling DJ, Hemmati-Brivanlou A, Derynck R. 2001. Regulation of Smad degradation and activity by Smurf2, an E3 ubiquitin ligase. Proc Natl Acad Sci 98:974-979.

Zheng W, Magid MS, Kramer EE, Chen Y-T. 1996. Follicle-stimulating hormone receptor is expressed in human ovarian surface epithelium and fallopian tube. Am J Path 148:47-53.

Zheng W, Lu JJ, Luo F, Zheng Y, Feng Y, Feliz JC, Lauchlan SC, Pike MC. 2000. Ovarian epithelial tumor growth promotion by follicle-stimulating hormone and inhibition of the effect by luteinizing hormone. Gynecol Oncol 76:80-88.

Zhou L, Leung BS. 1992. Growth regulation of ovarian cancer cells by epidermal growth factor and transforming growth factors α and ß. Biochim Biophys Acta 1180:130-136.

Zhou S, Zawel L, Lengauer C, Kinzler KW, Volgelstein B. 1998. Characterization of human FAST-1, a TGF beta and activin signal transducer. Mol Cell 2:121-127.

Zhu H, Kasvak P, Abdollah S, Wrana JL, Thomsen GH. 1999. A Smad ubiquitin ligase targets the BMP pathway and affects embryonic pattern formation. Nature 400:687-693.

Zhu Y, Richardson JA, Parada LF, Graff JM. 1998. Smad3 mutant mice develop metastatic colorectal cancer. Cell 94:703-714.