THE EFFECTS OF THE FEMALE REPRODUCTIVE HORMONES ON OVARIAN CANCER INITIATION AND PROGRESSION IN A TRANSGENIC MOUSE MODEL OF THE DISEASE

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Authorization

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Contributions of Co-authors

All of the studies contained in this thesis were conducted under the supervision of Dr. Barbara Vanderhyden. Unless otherwise specified, all of the experiments were performed by Laura Laviolette. Laura Laviolette designed the experiments, interpreted and analysed the data and wrote the manuscripts, with supervision and revisions from Dr. Barbara Vanderhyden.

Chapter 2
Title: 17β-estradiol accelerates tumour onset and decreases survival in a transgenic mouse model of ovarian cancer

Journal: Endocrinology (2010 Mar;151(3):929-38)

Authors: Laura A. Laviolette, Kenneth Garson, Elizabeth A. Macdonald, Mary K. Senterman, Kerri Courville, Colleen A. Crane, and Barbara C. Vanderhyden

Kenneth Garson and Kerri Courville generated the tgCAG-LS-TAg transgenic mouse model. Elizabeth Macdonald performed the radioimmunoassays. Mary Senterman assessed the histology of the mouse ovarian tumours. Colleen Crane assisted with the embedding, sectioning and IHC staining of the tumours.

Chapter 3

Title: 17β-estradiol accelerates ovarian tumour progression *in vivo* through the upregulation of Greb1

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Authors: Laura A. Laviolette, Kendra M. Hodgkinson, Neha Minhas, Carolina Perez-Iratxeta, and Barbara C. Vanderhyden

Kendra Hodgkinson performed the survival study in the ovariectomized immunodeficient mice and performed the Q-PCR validation of the microarray targets. Neha Minhas performed the *in vitro* proliferation assay. Carolina Perez- Iratxeta analysed the microarray data.

Chapter 4

Title: Induction of a menopausal state alters the growth and histology of ovarian tumours in a mouse model of ovarian cancer

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Authors: Laura A. Laviolette, Jean-François Ethier, Mary K. Senterman, Patrick J. Devine and Barbara C. Vanderhyden

Jean-François Ethier performed the xenograft study in ovariectomized immunodeficient mice. Mary Senterman analyzed and diagnosed the histology of the mouse ovarian tumours. Patrick Devine assisted with the interpretation and analysis of results and provided the protocols for the mouse VCD treatments.

Abstract

Ovarian cancer is thought to be derived from the ovarian surface epithelium (OSE), but it is often diagnosed during the late stages and therefore the events that contribute to the initiation and progression of ovarian cancer are poorly defined. Epidemiological studies have indicated an association between the female reproductive hormones and ovarian cancer etiology, but the direct effects of 17β-estradiol (E2), progesterone (P4), luteinizing hormone (LH) and follicle stimulating hormone (FSH) on disease pathophysiology are not well understood.

A novel transgenic mouse model of ovarian cancer was generated that utilized the Cre/*loxP* system to inducibly express the oncogene SV40 large and small T-Antigen in the OSE. The tgCAG-LS-TAg mice developed poorly differentiated ovarian tumours with metastasis and ascites throughout the peritoneal space. Although P4 had no effect; E2 significantly accelerated disease progression in tgCAG-LS-TAg mice. The early onset of ovarian cancer was likely mediated by E2's ability to increase the areas of putative preneoplastic lesions in the OSE.

E2 also significantly decreased survival time in ovarian cancer cell xenografts. Microarray analysis of the tumours revealed that E2 mainly affects genes involved in angiogenesis and cellular differentiation, proliferation, and migration. These results suggest that E2 acts on the tumour microenvironment in addition to its direct effects on OSE and ovarian cancer cells.

In order to examine the role of the gonadotropins in ovarian cancer progression, the tgCAG-LS-TAg mice were treated with 4-vinylcyclohexene-diepoxide (VCD) to

induce menopause. Menopause slowed the progression of ovarian cancer due to a change in the histological subtype from poorly differentiated tumours to Sertoli tumours.

Using a transgenic mouse model, it was shown that E2 accelerated ovarian cancer progression, while P4 had little effect on the disease. Menopause (elevated levels of LH and FSH) altered the histological subtype of the ovarian tumours in the tgCAG-LS-TAg mouse model. These results emphasize the importance of generating animal models to accurately recapitulate human disease and utilizing these models to develop novel prevention and treatment strategies for women with ovarian cancer.

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List of Abbreviations and Chemical Formulae

>	greater than
°C	degrees Celsius
3xpolyA	three downstream polyadenylation signals
12L:12D	12 hours light and 12 hours dark
18S	18S ribosomal RNA
α	alpha
α-MEM	alpha minimum essential medium
ABI	Applied Biosystems
AdCre	adenovirus expressing Cre recombinase
Add1	adducin 1 (alpha)
AdGFP	adenovirus expressing eGFP
Akt	v-akt murine thymoma viral oncogene homolog 1
ANOVA	analysis of variance
Apc	adenomatous polyposis coli
ATCC	American Type Culture Collection
β	beta
Bcl-2	B-cell CLL/lymphoma 2
β-gal	β-galactosidase
β-geo	a fusion of β-galactosidase and neomycin phosphotransferase genes
Bmp6	bone morphogenetic protein 6
bp	base pair(s)
Brca1	breast cancer 1 early onset
Brca2	breast cancer 2 early onset
Ca^{2+}	calcium
CAG	cytomegalovirus early enhancer and chicken beta-actin
CD45R	leukocyte common antigen
Cdh2	N-cadherin
Cdk	cyclin-dependent kinase
cDNA	complimentary DNA
CK19	cytokeratin 19
cm	centimetre(s)
c-myc	v-myc myelocytomatosis viral oncogene homolog (avian)
CMV	cytomegalovirus
CO_2	carbon dioxide
Co-AC	co-activator proteins
Cox-1	cyclooxygenase-1
Cox-2	cyclooxygenase-2
Cre	cyclization (Cre) recombinase
Ctnnb1	catenin (cadherin-associated protein) beta 1

Cyp11a1	cholesterol side-chain cleavage enzyme
DAB	diaminobenzidine
Dab2	disabled homolog 2
DBS	donor bovine serum
DMBA	7,12-dimethylbenz[a]anthracene
DMEM	Dulbecco's Modified Eagle Medium
DMEM/F12	Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12
DNA	deoxyribonucleic acid
E2	17β-estradiol
Ednra	endothelin receptor type A
EOC	epithelial ovarian cancer
eGFP	enhanced green fluorescent protein
ER	estrogen receptor(s)
ERα	estrogen receptor alpha
ERβ	estrogen receptor beta
ErbB-2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)
ERE	estrogen response element(s)
et al	et alii
Fas	Fas (TNF receptor superfamily, member 6)
FasL	Fas ligand (TNF superfamily, member 6)
FBS	fetal bovine serum
Fgf7	fibroblast growth factor 7
Fig.	Figure
floxed	flanked by *loxP* sites
Flp	flippase
Foxl2	forkhead box protein L2
Foxo3a	forkhead box O3
FRT	flippase recognition target
FSH	follicle stimulating hormone
FSHR	follicle stimulating hormone receptor
FWD	forward
g	gravitational
g	gram(s)
Gapdh	glyceraldehyde-3-phosphate dehydrogenase
gcd	germ cell deficient
GO	gene ontology
GOA	gene ontology annotation
Gpr3	G-protein-coupled receptor 3
GPR30	G protein-coupled receptor 30
Greb1	gene regulated by estrogen in breast cancer 1

H_2O_2	hydrogen peroxide
H&E	hemotoxylin and eosin
HIF-1α	hypoxia-inducible factor 1 alpha
HRP	horseradish peroxidise
hrs	hours
HRT	hormone replacement therapy
IGF-I	insulin-like growth factor 1
IHC	immunohistochemistry
IL-6	interleukin 6
IP	intraperitoneal
IRES	internal ribosome entry site
IRMA	immunoradiometric assay
ITSS	insulin–transferrin–sodium selenite solution
JNK	c-Jun N-terminal kinase
Kdr	vascular endothelial growth factor receptor 2
kg	killogram(s)
Kit	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
Kitl	kit ligand
Kras	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
KRT7	keratin 7
LacZ	β-galactosidase
LH	luteinizing hormone
LHR	luteinizing hormone receptor
LMP	low melting point
logFC	logarithmic fold change
LoxP	locus of X-over P1
µg	microgram(s)
µL	microlitre(s)
µm	micrometer(s)
µM	micromolar
MAPK	mitogen-activated protein kinase(s)
MAS	mouse ovarian cancer ascites cell line
MASC1	mouse ovarian cancer ascites control cell line 1
MASC2	mouse ovarian cancer ascites control cell line 2
MASE1	mouse ovarian cancer ascites estrogen cell line 1
MASE2	mouse ovarian cancer ascites estrogen cell line 2
MASP1	mouse ovarian cancer ascites progesterone cell line 1
MASP2	mouse ovarian cancer ascites progesterone cell line 2
Met	met proto-oncogene
mg	milligram(s)

MGI	mouse genome informatics
min	minute(s)
MISIIR	Mullerian inhibitory substance type II receptor
mL	millilitre(s)
MMP-2	matrix metalloproteinase 2
MMP-9	matrix metalloproteinase 9
mOSE	mouse ovarian surface epithelium
mRNA	messenger RNA
Msy2	Y box protein 2
NaCl	sodium chloride
NCBI	National Center for Biotechnology Information
NCoR	nuclear receptor co-repressor
ND	non-detectable
ng	nanogram(s)
nM	nanomolar
OSE	ovarian surface epithelium
OVX	ovariectomy
P	probability
P4	progesterone
p53	tumour protein p53
pan-CK	pan-cytokeratins
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pfu	plaque forming unit(s)
pg	picogram(s)
PgR	progesterone receptor
PI3K	phosphoinositide-3-kinase(s)
PIK3CA	phosphoinositide-3-kinase, catalytic, alpha polypeptide
PLAP	placental alkaline phosphatase
Plxna3	plexin A3
polyA	polyadenylation
PR	progesterone receptor
PRA	progesterone receptor A
PRB	progesterone receptor B
Pten	phosphatase and tensin homolog
Ptgs1	cyclooxygenase-1
Q-PCR	quantitative reverse transcriptase polymerase chain reaction
Rap-2	RAP2A, member of RAS oncogene family
Rb1	retinoblastoma 1
REV	reverse
RIA	radioimmunoassay

RMA	log scale robust multi-array analysis
RNA	ribonucleic acid
Rnps1	RNA binding protein S1, serine-rich domain
rpm	revolutions per minute
RR	relative risk
RT-PCR	reverse transcriptase polymerase chain reaction
S100α	S100 calcium binding protein A1
SCID	severe combined immunodeficiency disease
SDF-1	stromal-cell derived factor 1
SEM	standard error of the mean
Sgpp1	sphingosine-1-phosphate phosphatase 1
SKIIP	SKI interacting protein
Smad4	SMAD family member 4
SRC	steroid receptor coactivator
SRC1	steroid receptor coactivator 1
SRC2	steroid receptor coactivator 2
SRC3	steroid receptor coactivator 3
Stat3	signal transducer and activator of transcription-3
Stc2	Stanniocalcin 2
SV40 TAg	Simian vacuolating virus 40 large and small T antigens
TAF4b	TAF4B RNA polymerase II
tgCAG-LS-TAg	transgenic mouse with the CAG promoter driving expression of a stop sequence flanked by *loxP* sites and the SV40 TAg oncogene
TGFβ-1	transforming growth factor, beta 1
TGFβ2	transforming growth factor, beta 2
TGFβ3	transforming growth factor, beta 3
TP53	tumour protein p53
Tsc2	tuberous sclerosis 2
TVA	receptor for subgroup-A avian leukosis virus
U	unit(s)
VCD	4-vinylcyclohexene diepoxide
VEGF	vascular endothelial growth factor A
VEGF-A	vascular endothelial growth factor A
vs.	versus
v/v	volume for volume
Wnt	wingless-type MMTV integration site family

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Chapter 1: Introduction

Literature Review

Ovarian cancer

Incidence and mortality

Ovarian cancer is diagnosed in an estimated 2,600 Canadian women yearly, while 1,750 women die of the disease each year, making it the most lethal of the gynaeocologic malignancies (Canadian Cancer Society, Statistics Canada et al. 2010). Although the survival rates for patients range from 28% to 94% depending on the histological subtype and stage, the overall 5-year survival rate is only 46% (Chan, Cheung et al. 2006; Jemal, Siegel et al. 2010). The gravity of the disease is attributed to a number of factors including the lack of methods for early detection, the advanced stage at diagnosis (62% of patients are diagnosed with metastatic disease (Jemal, Siegel et al. 2010), the dearth of effective therapeutics against the disease, and disease recurrence which is often chemotherapy resistant and difficult to treat. For these reasons there is a need for a better understanding of the etiology of ovarian cancer, and in turn the development of more effective diagnostics and treatments against the disease.

Standard of treatment for ovarian cancer

The current standard of treatment for ovarian cancer is surgery and adjuvant chemotherapy (Yap, Carden et al. 2009). Cytoreductive surgery is crucial in the management of ovarian cancer, since patients who have less than 2 cm of residual disease

following surgical debulking survive 40-45 months, whereas patients who are suboptimally debulked (greater than 2 cm residual disease) have a survival time of only 12-16 months (Mutch 2002; Yap, Carden et al. 2009). Chemotherapeutics are used as adjuvants in ovarian cancer treatment and are given prior and/or after surgery. The first-line chemotherapy is a combination of platinum-paclitaxel, which achieves an overall response rate of up to 80% (McGuire, Hoskins et al. 1996; Piccart, Bertelsen et al. 2000; Biagi and Eisenhauer 2003; Ozols, Bundy et al. 2003; Yap, Carden et al. 2009). Although women respond well to surgery and chemotherapy initially, over 70% of women with advanced stage disease (stage III and IV) develop recurrent ovarian cancer that is often resistant to similar chemotherapeutic drugs and eventually fatal (Piccart, Bertelsen et al. 2000; Ozols, Bundy et al. 2003; Armstrong, Bundy et al. 2006). The high rate of disease recurrence, despite successful first-line treatment, remains one of the greatest challenges for treating women with ovarian cancer.

The etiology of ovarian cancer

Tissues of origin of epithelial ovarian cancers

The etiology of ovarian cancer remains poorly understood due to the high proportion of women diagnosed with advanced stage disease (Jemal, Siegel et al. 2010). It is believed that approximately 90% of epithelial ovarian cancers (EOCs) originate from the ovarian surface epithelium (OSE), which is a single layer of simple squamous to cuboidal cells that lines the entire surface of the ovary and is separated from the underlying ovarian stroma via a basement membrane (Auersperg, Wong et al. 2001). During development, the OSE and the extraovarian mesothelium originate from the same

tissue, the celomic epithelium. In addition to covering the ovaries, the celomic epithelium invades into the ovarian cortex, forming cords which eventually become the granulosa cells in the primordial follicles. The celomic epithelium also gives rise to the Mullerian ducts, which eventually develop into the oviductal (fallopian tube), endometrial, and endocervical epithelia (Auersperg, Wong et al. 2001). Unlike most epithelial cells, the OSE cells are poorly-differentiated, uncommitted cells expressing markers of both mesenchymal (vimentin) and epithelial cells (keratin 7) (Auersperg, Wong et al. 2001). They retain the ability to alter their state of differentiation, becoming more mesenchymal during repair of the ovulatory wound and undergoing Mullerian differentiation during ovarian tumourigenesis. The OSE layer is disrupted during each ovulation when the oocyte is extruded from within the ovary. Immediately following ovulation there is rapid proliferation and reorganization of the OSE to close the ovulatory wound, which may contribute to transformation of the OSE cells (Auersperg, Wong et al. 2001).

Despite our poor understanding of the early events in ovarian cancer initiation, putative preneoplastic lesions for EOC have been described and include 1) proliferative metaplasia and 2) cytologic atypia in the OSE, OSE invaginations and/or epithelial inclusion cysts (Bell 2005). The ovarian precursor lesions are believed to occur with increasing frequency as women age and possibly due to inappropriate healing of the OSE layer following ovulation (Heller, Murphy et al. 2005). More inclusion cysts are found in women with familial ovarian cancer (*BRCA1*/*BRCA2* mutation carriers) (Werness, Afify et al. 1999), in the contralateral ovary of women who have ovarian cancer (Mittal, Zeleniuch-Jacquotte et al. 1993), and in post-menopausal women (Heller, Murphy et al.

2005). Inclusion cysts derived from OSE cells undergo Mullerian epithelial differentiation (usually to a serous phenotype) likely due to their proximity to follicular fluid and stroma-derived growth factors (Feeley and Wells 2001).

Recent evidence suggests that the secretory epithelial cells of the fallopian tube may also give rise to ovarian cancer, specifically the serous histological subtype of EOCs, in women with *BRCA1/BRCA2* mutations or a strong family history for the disease (Agoff, Mendelin et al. 2002; Medeiros, Muto et al. 2006; Callahan, Crum et al. 2007; Crum, Drapkin et al. 2007; Lee, Miron et al. 2007). Putative precursor lesions, such as dysplasia and hyperplasia, and early tubal intraepithelial carcinomas have been detected in the tubal fimbria of women undergoing prophylactic salpingo-oophorectomy due to their elevated risk (Piek, van Diest et al. 2001; Medeiros, Muto et al. 2006; Kindelberger, Lee et al. 2007; Lee, Miron et al. 2007). It is still unclear whether or not serous EOCs occur in the fallopian tubes of non-mutation carriers and what proportion of all EOCs initiate in the fallopian tube compared to OSE cells and inclusion cysts.

Histological subtypes

The human ovary is composed of follicles, oocytes (germ cells) and stroma, which are separated from the OSE cells by a layer of connective tissue called the tunica albuginea. Ovarian cancers are broadly divided into four groups: epithelial (93% of all cases), germ cell (2.5%), sarcomas (2.7%) and sex cord stromal tumours (1.8%) (Chan, Cheung et al. 2006). Germ cell tumours arise from the female germ cells and are most commonly diagnosed in children and young woman (Colombo, Peiretti et al. 2009). Ovarian sarcomas occur infrequently and are usually diagnosed during the late stages,

therefore the exact tissue of origin is not yet known (Brown, Stewart et al. 2004). However, it is hypothesized that ovarian sarcomas arise from areas of endometriosis (Anderson, Turner et al. 1987). Sex cord stromal tumours occur following the malignant transformation of granulosa cells, thecal cells or ovarian stromal cells (Colombo, Parma et al. 2007).

Epithelial ovarian cancers (EOCs) are the most common type of ovarian cancers representing approximately 93% of all cases and will be the main type of ovarian cancer discussed in this thesis. EOCs are believed to be derived from the OSE and/or the fallopian tube and can be further divided into four histological subtypes: serous, endometrioid, mucinous, and clear cell (Cannistra 2004). Each histological subtype has unique morphological and genetic characteristics (Bell 2005). The serous subtype is the most common EOC (approximately 80% of cases) and is distinguished by its formation of glandular and papillary structures reminiscent of fallopian tube epithelium (Auersperg, Wong et al. 2001) (Figure 1). Serous ovarian tumours often contain mutations in *TP53* and dysfunction of BRCA1 and/or BRCA2 (Geisler, Hatterman-Zogg et al. 2002; Hilton, Geisler et al. 2002; Ahmed, Etemadmoghadam et al. 2010; Bernardini, Baba et al. 2010). Endometrioid tumours resemble uterine epithelium with *PTEN*, *CTNNB1*, and *TP53* mutations being the most frequent genetic lesions (Obata, Morland et al. 1998; Palacios and Gamallo 1998; Wu, Zhai et al. 2001; Okuda, Otsuka et al. 2003). Mucinous ovarian tumours take on phenotypic characteristics of gastrointestinal or endocervical epithelium and are associated with mutations in *KRAS* (Gemignani, Schlaerth et al. 2003; Dobrzycka, Terlikowski et al. 2009). Clear cell epithelial ovarian cancers resemble

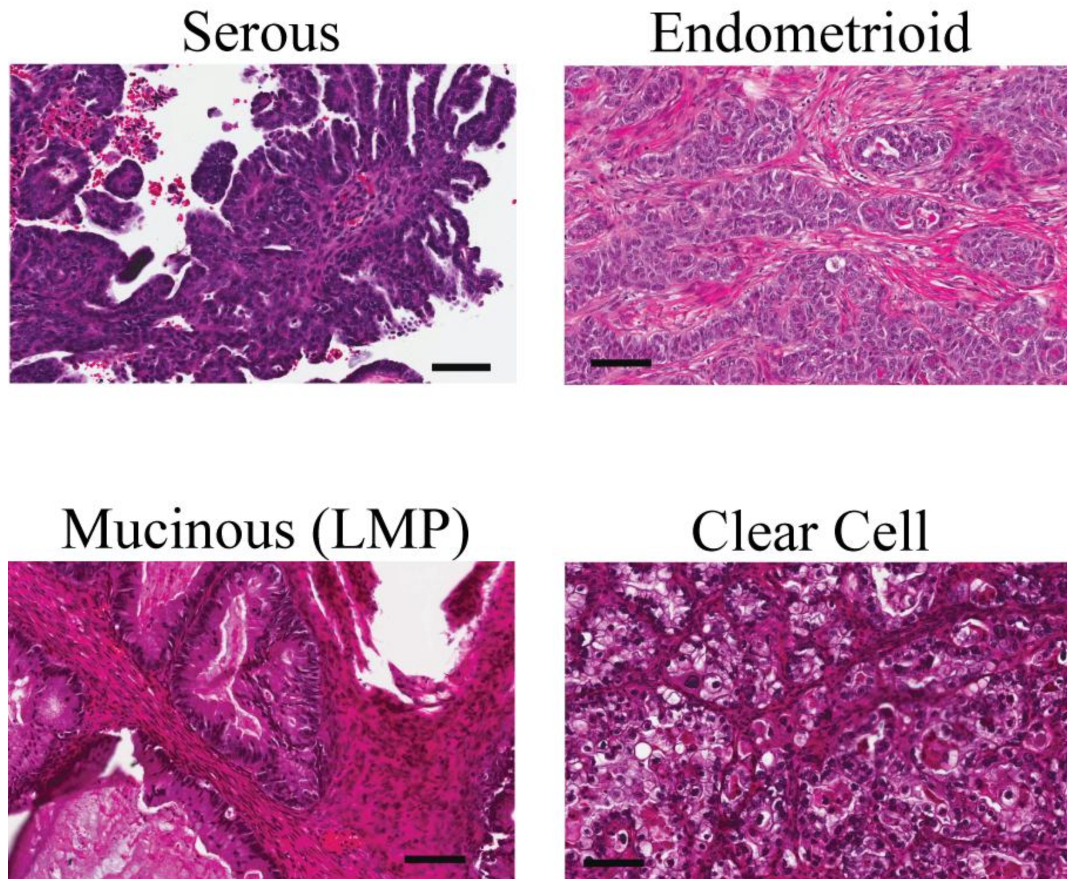


Figure 1. The four histological subtypes of epithelial ovarian carcinomas. Serous tumours resemble the fallopian tube epithelium; endometrioid tumours have characteristics of uterine epithelium; mucinous tumours have the morphology of gastorintestinal or endocervical epithelium; clear cell tumours take on a mesonephros-like appearance (scale bars represent 100 µm; LMP – low malignant potential). These images were provided courtesy of Colleen Crane and the Ottawa Ovarian Cancer Tumour Bank.

mesonephros and are associated with activating mutations of *PIK3CA* (Auersperg, Wong et al. 2001; Kuo, Mao et al. 2009). EOCs are different from most solid tumours because they become more epithelial as they progress; appearing more differentiated than the uncommitted OSE cells from which they originate (Auersperg, Wong et al. 2001).

Progression of ovarian cancer

Ovarian cancer has a high incidence of metastases which is partially attributed to the ease with which exfoliated tumour cells are transported via the peritoneal fluid to other tissues. These cells attach to the peritoneum and mesothelial linings of the pelvic and abdominal organs, such as the omentum, the liver, the intestinal mesentery and the diaphragm. In addition, ovarian cancer can metastasize, via direct extension, to adjacent tissues, such as the uterus, fallopian tubes, and the broad ligament. Ascites, which is an accumulation of fluid and cancer cells, is also associated with peritoneal spread of the disease (reviewed by (Naora and Montell 2005)). The staging system developed by the International Federation of Gynecology and Obstetrics ranges from Stage I to IV depending on the extent of disease spread (Cannistra 2004). Just over 30% of patients are diagnosed with Stage I and II ovarian cancers, which is defined as disease limited to the ovaries, pelvic organs and ascites (Cannistra 2004; Chan, Cheung et al. 2006). Women diagnosed in these early stages have a 5-year survival rate of approximately 80% (Auersperg, Wong et al. 2001). Most women (68%) have Stage III or IV disease at diagnosis which includes spread of tumours beyond the pelvis to distant organs such as the omentum and liver, and gross tumour deposits ≥ 2cm in diameter (Cannistra 2004).

Ovarian cancer patients diagnosed with advanced stage disease (Stage III and IV) only have a 30% 5-year survival rate (Chan, Cheung et al. 2006).

Theories for the origin of epithelial ovarian cancer

Theory of incessant ovulation

The theory of incessant ovulation postulates that ovulation is a major contributing factor for the initiation of EOC because of the repeated bouts of wounding and proliferation in the OSE, which may make it more susceptible for transformation (Fathalla 1971). Unlike most animals that only ovulate when a male is present to fertilize the egg, women undergo uninterrupted ovulations throughout their reproductive lifetimes (Fathalla 1971). Epidemiological studies support the theory of incessant ovulation - women who use combined oral contraceptives (Beral, Hannaford et al. 1988; Ness, Grisso et al. 2000; Beral, Doll et al. 2008) or who are multiparous (Risch, Weiss et al. 1983; Moorman, Calingaert et al. 2008) have a decreased risk of developing ovarian cancer, likely due to fewer lifetime ovulations. Although most studies would suggest that incessant ovulation increases the risk for developing ovarian cancer due to the ovulatory wound and repair, there are other factors involved in ovulation which may also contribute, for example, the hormonal changes associated with folliculogenesis, the growth factors present within the follicular fluid, and the inflammation associated with the wound.

Gonadotropin theory

The gonadotropin theory of ovarian carcinogenesis suggests that the high levels of follicle stimulating hormone (FSH) and luteinizing hormone (LH) present during the menopausal years contribute to the development of ovarian cancer by stimulating OSE cell proliferation leading to malignant transformation (Cramer and Welch 1983). The higher incidence of ovarian cancer in peri- and post-menopausal women supports the theory that elevated serum gonadotropins play a role in the etiology of the disease (Jemal, Siegel et al. 2008).

Risk factors for the development of ovarian cancer

Mutations in BRCA1 and BRCA2

A strong family history of ovarian cancer is the leading risk factor for developing the disease (Cannistra 2004). Hereditary germline mutations in the tumour suppressor genes *BRCA1* and *BRCA2* account for only approximately 15% of EOC cases (Pal, Permuth-Wey et al. 2005), however the lifetime risk of developing EOC is between 40 and 65% for women who have a mutation in *BRCA1* (Ford, Easton et al. 1994; Antoniou, Pharoah et al. 2003). This elevated risk makes mutations in *BRCA1* and *BRCA2* the most predictive genetic risk factor for the disease.

Women with mutations in *BRCA1* and *BRCA2* are also at an increased risk of developing breast cancer (Ford, Easton et al. 1994; Ford, Easton et al. 1998). While it is still not known why germline mutations in *BRCA1* mainly affect cancers of the ovary and breast, it is hypothesized that this could be due to alterations in steroid hormone (estrogen) signalling. For example, inactivation of BRCA1 in human granulosa cells

results in increased expression of aromatase, the rate limiting enzyme for the conversion of testosterone to 17β-estradiol (E2) (Hu, Ghosh et al. 2005). The expression of BRCA1 may be directly affected by steroid hormones since it is highly expressed in mouse mammary tissue during pregnancy and when mice are treated with exogenous E2 and progesterone (P4) (Lane, Deng et al. 1995; Marquis, Rajan et al. 1995). This increase in BRCA1 expression may be a direct effect of E2, or may result indirectly due to rapid proliferation of mammary cells during pregnancy and in response to steroid hormones. However, *BRCA1* does have putative estrogen response elements and estrogen receptor α (ERα) can be recruited to the *BRCA1* promoter following E2 treatment, suggesting that estrogens may have direct effects on BRCA1 expression (Jeffy, Hockings et al. 2005; Gorski, Kennedy et al. 2009). Despite the evidence for an association between estrogens and BRCA1 expression, most breast cancers that arise in *BRCA1* mutation carriers are estrogen receptor negative (Gorski, Kennedy et al. 2009). Therefore, the role of estrogens in the development of breast and ovarian cancers in women with germline *BRCA1* mutations is still poorly understood.

Menopause

Post-menopausal ovaries are devoid of germ cells and are mainly composed of stroma and epithelial inclusion cysts, but also retain the intact OSE layer. Once ovaries are depleted of follicles and oocytes, they lose their ability to produce the reproductive steroid hormones (Perheentupa and Huhtaniemi 2009). Consequently, the pituitary produces elevated levels of the gonadotropins (LH and FSH) which are believed to stimulate proliferation of the OSE cells, thus contributing to the initiation of ovarian

tumourigenesis (Cramer and Welch 1983; Davies, Finnigan et al. 1999). Ovarian cancer incidence increases during the peri- and post-menopausal years, with approximately 78% of all cases diagnosed in women aged 50 years or older (Chan, Cheung et al. 2006). Moreover, women diagnosed prior to age 50 have a more favourable 5-year survival rate of 70% compared to only 42% in those diagnosed during and after menopause ($\geq$ 50 years) (Chan, Cheung et al. 2006).

Hormone replacement therapy

After menopause, when endogenous levels of the reproductive steroid hormones are low, women can obtain exogenous hormones via hormone replacement therapy (HRT). HRT is composed of synthetic steroid hormones, usually combinations of both estrogens and progestins, although occasionally just one or the other. Epidemiological studies have revealed that HRT use increases the risk of developing ovarian cancer (Lacey, Mink et al. 2002; Riman, Dickman et al. 2002; Glud, Kjaer et al. 2004; Beral, Bull et al. 2007). One prospective study involving almost one million women in the United Kingdom (UK), found that the relative risk (RR) for current users of HRT was increased to 1.2 compared to never-users (Beral, Bull et al. 2007). HRT use alone accounted for approximately 1300 additional cases and 1000 ovarian cancer deaths in just 15 years (Beral, Bull et al. 2007). Estrogen-only regimens, current-use, and long-term use (greater than 5 years) confer the highest risk (Lacey, Mink et al. 2002; Glud, Kjaer et al. 2004; Beral, Bull et al. 2007). Moreover, HRT use is strongly linked to the serous histological subtype of EOC (Beral, Bull et al. 2007).

Similarly to ovarian cancer, HRT use increases the risk of developing breast cancer. Using the same cohort of a million women in the UK, researchers found that current users of HRT were more likely to develop breast cancer than never users (adjusted RR of 1.66) (Beral 2003). Interestingly, the risk of death was similar for both breast and ovarian cancer patients who were diagnosed while using HRT compared to never users (RR = 1.22; RR = 1.23, respectively) (Beral 2003; Beral, Bull et al. 2007). Although the risk of developing breast cancer while using HRT is higher than ovarian cancer, treatments for hormone responsive tumours are much more effective in women with breast cancer. Anti-estrogen therapies, such as the ERα antagonist tamoxifen, are very effective for the treatment of hormone receptor positive breast tumours (which represent over two-thirds of all cases) (Prat and Baselga 2008). Tamoxifen therapy can reduce the occurrence and mortality of ERα positive, early stage breast cancers by over 30% (Prat and Baselga 2008). Similarly, ERα is expressed in a significant portion of ovarian tumours (25-86% depending on the study), but receptor expression does not correlate with prognosis in ovarian cancers (Issa, Lebeau et al. 2009). Surprisingly, endocrine therapies (such as tamoxifen) have not been very successful for the treatment of ovarian cancers (Perez-Gracia and Carrasco 2002; Garrett and Quinn 2008). The lack of response to endocrine therapy may be due to inappropriate selection of recipients, for example failing to choose patients based on ERα expression, small study sample sizes, or because endocrine therapies have mainly been tested in patients with late-stage, chemotherapy resistant disease (Perez-Gracia and Carrasco 2002). A better understanding of the effects of endocrine therapy in combination with chemotherapy and for the long term management of ovarian cancer is required.

Oral contraceptives

Epidemiological studies have indicated that combined oral contraceptive use (combinations of estrogens and progestins) reduces the risk of developing ovarian cancer by up to 40%. Moreover, this protection is long-lasting and may persist for 15 years or more (Beral, Hannaford et al. 1988; Ness, Grisso et al. 2000; Deligeoroglou, Michailidis et al. 2003; Beral, Doll et al. 2008). Although it is generally accepted that oral contraceptives decrease the risk of developing EOC by reducing the total number of lifetime ovulations, the direct effect of the hormones on the OSE cells remains unclear.

Pregnancy

Similar to the use of oral contraceptives, epidemiologic studies have shown that pregnancy decreases the risk of developing EOC, likely as a result of decreasing the total number of lifetime ovulations. The risk is further decreased with each additional child and the reduction in risk is similar across all of the histological subtypes (Whittemore, Harris et al. 1992; Kurian, Balise et al. 2005; Sueblinvong and Carney 2009). Pregnancy is associated with a physiological change in hormone levels that includes progestins and estrogens being elevated for the duration of the pregnancy. Whether the alterations in hormone levels also contribute to the risk of EOC in pregnant women is not yet well characterized.

Reproductive hormones and ovarian cancer

While there is strong epidemiological evidence supporting an association between female reproductive hormones and ovarian cancer, the mechanisms through which E2, P4, LH, and FSH affect ovarian cancer onset and progression are not well-understood. Until recently, the lack of transgenic mouse models of ovarian cancer limited the study of disease modulators, such as hormones, on *in situ* disease progression. Despite this, previous basic research studies have shown that the reproductive hormones can alter OSE cell proliferation and morphology and affect ovarian cancer growth.

17β-estradiol

Estrogen signalling is critical for the normal function of many tissues including the reproductive tract, mammary glands, central nervous system, skeletal system and cardiovascular system (Hewitt, Harrell et al. 2005). The biological effects of estrogens are mediated via the classical genomic signalling pathway and the non-genomic pathway (Figure 2), eliciting changes in several estrogen responsive genes which modulate many cellular functions, such as proliferation, migration, adhesion and differentiation (Segars and Driggers 2002; Weihua, Andersson et al. 2003). The classical estrogen signalling pathway involves binding of E2 to the estrogen receptors α (ERα) and/or β (ERβ), which act as ligand-dependent transcription factors in the nucleus (Segars and Driggers 2002). ERα and ERβ modulate gene transcription by recognizing and binding DNA at specific

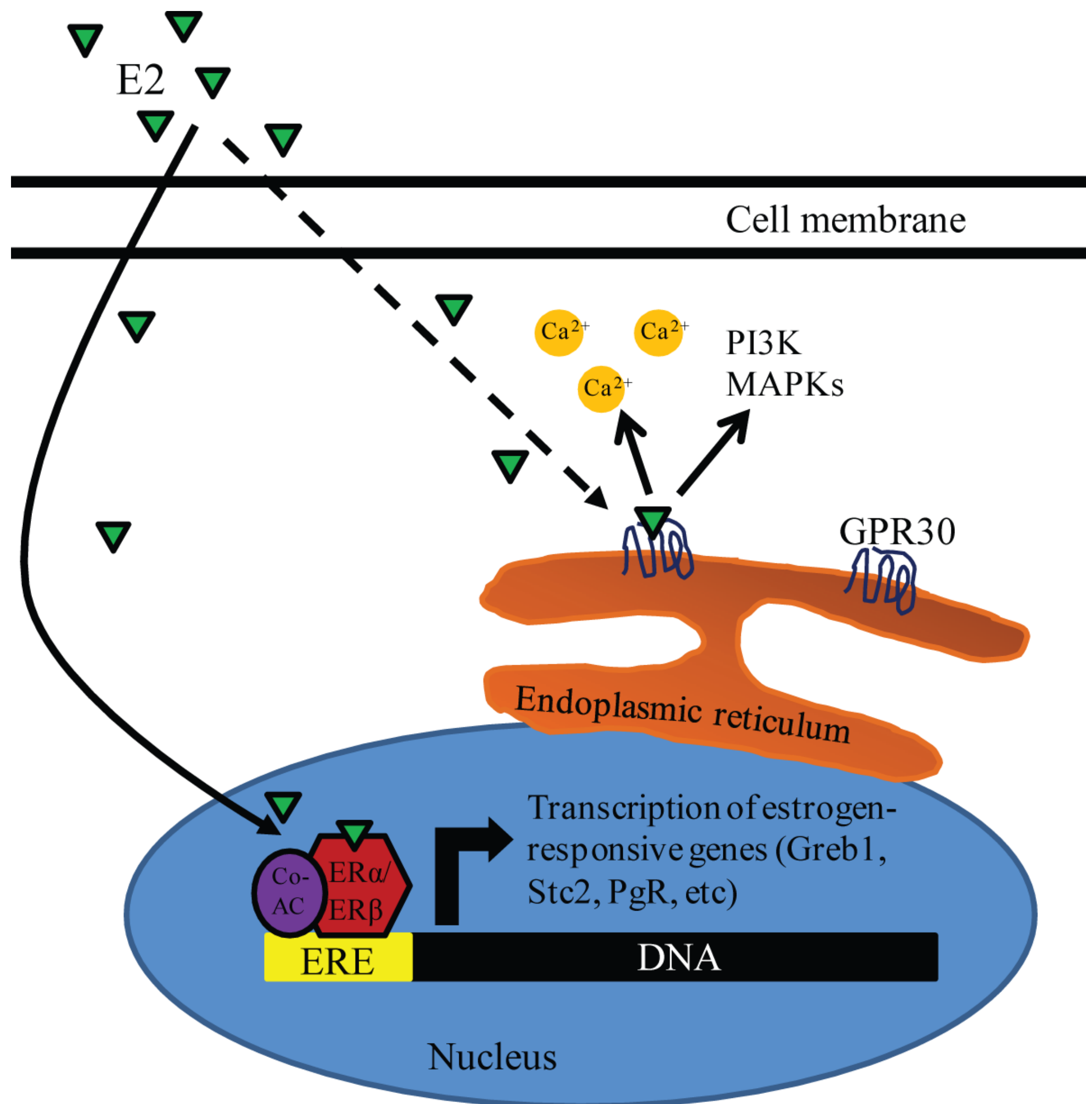


Figure 2. The genomic and non-genomic E2 signalling pathways. E2 enters the cell via passive diffusion across the cell membrane (solid arrow). E2 is able to enter the cell nucleus where it binds the estrogen receptors ERα and/or ERβ. Following ligand binding, the ERs associate with co-activator proteins (Co-AC) which allow ERs to bind to specific DNA sequences known as estrogen response elements (EREs). The ligand-bound ERs induce the transcription of estrogen-responsive genes such as Greb1, PgR and Stc2. The non-genomic signalling pathway (dotted arrow) involves E2 binding to the G protein-coupled receptor GPR30, located in the endoplasmic reticulum. Following ligand binding and activation, GPR30 mediates rapid changes in intracellular calcium (Ca^{2+}) and activates PI3K and MAPK signalling. Adapted from ER Prossnitz et al., *Annu Rev Physiol* 70, 165-190 (2008) and SC Hewitt et al., *Annu Rev Physiol* 67, 285-308 (2005).

sites, known as estrogen response elements (EREs). There are 3,665 unique estrogen receptor binding sites in the human genome, suggesting that the effects of estrogens are diverse and may be different depending on the cell type and the cellular microenvironment (Carroll, Meyer et al. 2006). The actions of ERα and ERβ are controlled by their association with co-activators, such as steroid receptor coactivator (SRC) family members SRC1, SRC2, SRC3, and co-repressors, such as nuclear receptor co-repressor (NCoR), in protein complexes (Segars and Driggers 2002; Matthews and Gustafsson 2003).

The non-classical estrogen receptor is the G protein-coupled receptor, GPR30, which is localized to the endoplastic reticulum and is responsible for rapid non-genomic estrogen signalling events (Revankar, Cimino et al. 2005). E2 directly binds GPR30, resulting in the rapid mobilization of intracellular calcium and the activation of PI3K and MAPK signalling pathways (Revankar, Cimino et al. 2005; Prossnitz, Arterburn et al. 2008). It is likely that the normal and pathological actions of E2 are modulated by GPR30, in addition to the classical steroid receptors ERα and ERβ.

Several *in vivo* and *in vitro* studies have been performed to investigate the direct effects of E2 on the ovary. These studies corroborate the results obtained from epidemiological studies, suggesting that estrogens are involved in the initiation and progression of ovarian cancer. *In vitro* studies determined that E2 is able to stimulate the growth of OSE cells, which express both ERα and ERβ (Lau, K. M., Mok et al. 1999; Bai, Oliveros-Saunders et al. 2000; Murdoch and Van Kirk 2002; Syed, Ulinski et al. 2002; Lindgren, Cajander et al. 2004). Moreover, prolonged exposure to exogenous E2 *in vivo* altered the morphology of rabbit ovaries and increased both OSE cell proliferation

and the number of papillae covering the ovarian surface (Bai, Oliveros-Saunders et al. 2000). E2 treatment of mice, either alone or in combination with superovulation, significantly increased the areas of columnar and stratified OSE cells compared to untreated mice (Gotfredson and Murdoch 2007). E2 was also able to alter the morphology of rat OSE cells, inducing a thickening and pseudo-stratification in the OSE (Perniconi, Simoes Mde et al. 2008). Similar changes, specifically areas of surface papillomatosis, have also been observed in post-menopausal women ovaries following HRT use (Tok, Ertunc et al. 2006). E2 may stimulate growth of OSE cells via an increase in levels of interleukin 6 (IL-6), which is involved in cell cycle progression (Syed, Ulinski et al. 2002), or through E2's ability to inhibit apoptosis (Murdoch and Van Kirk 2002). All of these studies suggest that E2 is able to induce preneoplastic changes in the OSE cells that may make them more susceptible for oncogenic transformation.

In ovarian cancer cells, it is well known that E2 can act as a growth factor. E2 treatment *in vitro* caused increased proliferation in several human ovarian cancer cell lines, including OVCA 429, OVCA 432, BG-1, BR, PE04, PE01, PE06 and OVCAR-3 cells (Nash, Ozols et al. 1989; Langdon, Hawkes et al. 1990; Pavlik, Nelson et al. 1991; Langdon, Ritchie et al. 1993; Wimalasena, Meehan et al. 1993; Chien, Wang et al. 1994; Langdon, Hirst et al. 1994; Syed, Ulinski et al. 2002; Zhou, Luo et al. 2002). In most of these experiments, expression of ERα was required for enhanced proliferation, while ovarian cancer cells with no or low expression of ERα did not grow in response to exogenous E2 (Langdon, Hawkes et al. 1990; Chien, Wang et al. 1994; Langdon, Hirst et al. 1994). In addition to its proliferative effects, E2 decreased apoptosis of ovarian cancer cells *in vitro* (Mabuchi, Ohmichi et al. 2004), likely through an upregulation of

bcl-2 (Choi, Kang et al. 2001), indicating a pro-survival role of the hormone. Although the molecular mechanisms through which E2 acts on ovarian cancer cells are not well understood, some genes that are differentially expressed following E2 treatment include SDF-1 (Hall and Korach 2003), ezrin (Song, Fadiel et al. 2005), c-Myc (Sasaki, Hayakawa et al. 2008), IGF-I (Sasaki, Hayakawa et al. 2008), and fibulin-1 (Bardin, Moll et al. 2005), which all may affect the growth, survival, and migration of ovarian cancer cells. Despite strong evidence for a stimulatory effect of estrogens in ovarian cancer growth *in vitro*, xenograft studies indicated that E2 may suppress the growth of ovarian cancer *in vivo* (Langdon, Ritchie et al. 1993). These conflicting reports demonstrate the necessity for a thorough characterization of the effects of E2 on ovarian tumour initiation and progression *in situ.*

Progesterone

Experimental evidence suggests that P4 may guard against the malignant transformation of OSE cells *in vitro* by reducing the proliferation and inducing apoptosis in OSE cells that expresses the progesterone receptor (PR) (Lau, K. M., Mok et al. 1999; Syed, Ulinski et al. 2001; Wright, J. W., Toth-Fejel et al. 2002; Syed and Ho 2003; Lindgren, Cajander et al. 2004). Similarly, an *in vivo* study demonstrated an increase in the amount of apoptosis in the macaque OSE following long-term treatment with progestin (Rodriguez, Walmer et al. 1998). The protective effect of progestin *in vivo* was due to the resulting decreased expression of TGFβ-1 and increased expression of TGFβ2/3 in macaque ovaries.

Analogous to its effects in OSE cells, P4 reduced proliferation and increased apoptosis in ovarian cancer cells. Treatment of the ovarian cancer cell lines HOC-7 and OVCAR-3 with P4 *in vitro* reduced both cellular proliferation and soft agar colony formation (Blumenthal, Kardosh et al. 2003; Fauvet, Dufournet Etienne et al. 2006). The *in vitro* action of P4 on proliferation was mediated through a reduction of cyclin-dependent kinase and bcl-2 activity and an upregulation of caspase-3 activity (Blumenthal, Kardosh et al. 2003; Fauvet, Dufournet Etienne et al. 2006). Ovarian cancer cells treated with P4 also undergo apoptosis due to activation of the caspase-8-initiated Fas/FasL signalling pathway (Syed and Ho 2003).

Studies performed with ovarian cancer xenografts showed that *in vivo* treatment with exogenous P4 suppresses tumourigenesis and significantly prolongs survival (McDonnel, Van Kirk et al. 2003; McDonnel, Van Kirk et al. 2005). Interestingly, the increased survival was only seen when the mice were treated prophylactically with the hormone (McDonnel, Van Kirk et al. 2005). Contrary to the mouse experiments, a clinical trial showed that P4 was most effective on late stage ovarian cancer (Chen, X. and Feng 2003). Combining P4 and platinum-based chemotherapy to clinically treat EOC resulted in increased three year survival rates and decreased three year recurrence rates, but the effects were only observed in patients suffering from stage III and stage IV disease (Chen, X. and Feng 2003). All of these studies support the theory that P4 protects against ovarian cancer initiation and progression.

There is clearly a role for E2 and P4 in ovarian cancer initiation and progression, however the mechanisms have not been well-characterized. Moreover, there is a need to delineate the actions of the reproductive steroid hormones on OSE cells (tumour

initiation) vs. ovarian cancer cells (tumour progression) to fully understand the reproduction-associated ovarian cancer risk factors, such as hormone replacement therapy and oral contraceptives.

Luteinizing hormone

It is hypothesized that the higher incidence of ovarian cancer during the peri- and post-menopausal years is, in part, due to unopposed elevated serum gonadotropins which stimulate proliferation of OSE cells. Very little is currently known about the direct effects of LH on the OSE, but OSE cells do express the LH receptor (LHR), which is also present in a high proportion (40%) of ovarian carcinomas (Parrott, Doraiswamy et al. 2001; Choi, Wong et al. 2007). An *in vivo* study in mice indicated that exogenous LH increases the proliferation of OSE cells (Davies, Finnigan et al. 1999). LH treatment of the ovarian cancer cell line SKOV3 resulted in an upregulation of ErbB-2, a receptor tyrosine kinase that is often correlated with poor survival in cancers (Warrenfeltz, Lott et al. 2008). LH has also been shown to increase expression of the pro-angiogenic factor VEGF in EOC cells (Wang, Luo et al. 2002). *In vitro* treatment of ovarian cancer cell lines with LH caused an increase in invasion due to elevated expression of MMP-2 and MMP-9 (Choi, Choi et al. 2006). Moreover, LH may assist ovarian cancer cells to evade apoptosis, since concomitant treatment of HEY cells with LH and a Fas receptor agonist inhibited Fas-mediated apoptosis (Slot, de Boer-Brouwer et al. 2006).

Follicle stimulating hormone

FSH increases OSE and ovarian cancer cell proliferation, which is likely due to activation of the FSH receptor (FSHR), which is present in OSE cells, inclusion cysts and EOCs (Zheng, Lu et al. 2000; Parrott, Doraiswamy et al. 2001; Ji, Liu et al. 2004; Choi, Wong et al. 2007). FSH has been shown to increase proliferation and protect against cell death in OSE cells (Davies, Finnigan et al. 1999; Ji, Liu et al. 2004; Doyle and Donadeu 2009). However, when primary cultures of human OSE cells were treated with FSH, a proliferative effect was only observed in a subset of those tested, which may be attributed to variability in FSHR expression between samples (Edmondson, Monaghan et al. 2006). FSH stimulated growth of immortalized and transformed OSE cells through an activation of MAPK signalling (Choi, Kang et al. 2002).

SKOV-3 ovarian cancer cells exhibited enhanced proliferation, migration and Notch-1 signalling when treated with FSH (Park, Y. H., Kim et al. 2010). Studies indicate that the FSH-induced changes in gene expression are different in OSE cells vs. ovarian cancer cells and that FSH may be acting through neogenin and restin to accelerate growth of ovarian cancer (Ho, Lau et al. 2003). Other studies have implicated the PI3K/AKT signalling pathway in the growth and survival of FSH-treated ovarian cancer cells, because of an upregulation of downstream factors such as survivin, VEGF, COX-1 and COX-2 (Huang, Hua et al. 2008; Huang, Jin et al. 2010; Lau, M. T., Wong et al. 2010). Moreover, microarray analysis revealed the downregulation of tumour suppressor genes, such as *BRCA1* and *RB1*, in ovarian cancer cells treated with FSH, further suggesting that this hormone is involved in disease progression (Ji, Liu et al. 2004).

Despite the strong epidemiological and experimental support for the role of gonadotropins in ovarian cancer etiology, there are also some conflicting reports indicating that LH and FSH (either alone or in combination) induce apoptosis and decrease proliferation in OSE and ovarian cancer cells (Ivarsson, Sundfeldt et al. 2001; Wright, J. W., Toth-Fejel et al. 2002; Pon, Auersperg et al. 2005). Due to the deficiency of ovarian cancer and menopause models, the role of elevated serum gonadotropins in ovarian cancer initiation and progression has not been fully elucidated.

Mouse models of menopause

The incidence of ovarian cancer increases in peri- and post-menopausal women; however it remains unclear whether this is due to the structural changes that occur in the ovaries at that time or the consequent alterations in steroid and gonadotropic hormone levels. Mice are often used to model human physiology and disease. Follicle growth and ovulation, as well as the corresponding changes in hormonal levels are similar in mice and humans (Hong, Yen et al. 2010). The mouse estrus cycle lasts 4-5 days and consists of four stages, proestrus, estrus, metestrus and diestrus (Hong, Yen et al. 2010). Proestrus is equivalent to the follicular phase in women (high E2), estrus is the same as ovulation, metestrus corresponds to the luteal phase (high E2 and P4) and diestrus is similar to the late luteal phase (high P4) (Smith, Freeman et al. 1975; Schedin, Mitrenga et al. 2000; Wood, Fata et al. 2007; Hong, Yen et al. 2010). Despite the similar ovulatory cycles in mice and humans, menopause is not easily studied in mice because even at advanced age mice continue to have reproductive cycles, do no undergo a complete depletion of oocytes, and exhibit only slight changes in circulating levels of FSH, LH, E2

and P4 (Gee, Flurkey et al. 1983). The most frequently used model of menopause is the ovariectomized (OVX) mouse. While the OVX mouse model mimics the hormonal changes associated with menopause, including a reduction in E2 and P4 and an increase in LH and FSH (Gee, Flurkey et al. 1983), it is not useful for studying tumours that arise in the ovary.

Menopause can also be modelled in mice with ovarian germ cell deficiency due to mutations in *Kit*, *Kitl*, or *gcd* (Murphy 1972; Murphy and Beamer 1973; Ishimura, Matsuda et al. 1986; Duncan and Chada 1993). However, in these mice germ cell deficiency occurred embryonically therefore the mice never developed normal follicles and did not undergo a gradual depletion of oocytes with age, which precludes them from recapitulating the physiology of human menopause (Murphy 1972; Murphy and Beamer 1973; Ishimura, Matsuda et al. 1986; Duncan and Chada 1993). Moreover, the lack of germ cells in these mice eventually resulted in the formation of ovarian tubular adenomas (Murphy 1972; Murphy and Beamer 1973; Ishimura, Matsuda et al. 1986; Duncan and Chada 1993), a type of ovarian tumour that appears to have no parallel in humans.

Although menopause can modelled in mice with embryonic germ cell deficiencies, there are also genetic mouse models in which germ cell loss occurs postnatally, such as those with germline or ovarian-specific deficiencies in follistatin, androgen receptor, forkhead box protein L2 (Foxl2), forkhead box O3 (Foxo3a), TAF4B RNA polymerase II (TAF4b), tuberous sclerosis 2 (Tsc2), phosphatase and tensin homolog (PTEN), retinoblastoma (Rb), SMAD family member 4 (Smad4), G-protein coupled receptor 3 (Gpr3) or Y box protein 2 (Msy2) (Freiman, Albright et al. 2001; Castrillon, Miao et al. 2003; Jorgez, Klysik et al. 2004; Schmidt, Ovitt et al. 2004;

Ledent, Demeestere et al. 2005; Yang, J., Medvedev et al. 2005; Pangas, Li et al. 2006; Shiina, Matsumoto et al. 2006; Voronina, Lovasco et al. 2007; Andreu-Vieyra, Chen et al. 2008; Reddy, Liu et al. 2008; Adhikari, Flohr et al. 2009). Many of these models achieve germ cell loss by directly or indirectly perturbing hormone signalling pathways, precluding them from being used to study the effects of menopause-associated hormonal changes on ovarian tumour progression.

Several chemicals, such as cyclophosphamide (Plowchalk and Mattison 1991; Plowchalk and Mattison 1992; Desmeules and Devine 2006), DMBA (Johnson, Canning et al. 2004), and busulphan (Johnson, Canning et al. 2004; Tan, Yeh et al. 2010), have been shown to destroy rodent oocytes and follicles, however the ability of these chemicals to model the hormonal changes associated with menopause has not been well characterized. Recently, a novel mouse model of menopause was developed, which employed the industrial chemical 4-vinylcyclohexene diepoxide (VCD) to deplete female mice of their follicles at 3 months of age (Mayer, Devine et al. 2004). All of the VCD-treated mice were in persistent diestrus by 58 days after the initiation of VCD treatment (Mayer, Devine et al. 2004). Plasma samples collected from female mice 127 days after VCD treatment showed elevated levels of FSH and LH, reduced levels of progesterone, and undetectable amounts of estradiol (consistent with human menopause) (Mayer, Devine et al. 2004).

Since the discovery that VCD initiates a post-menopause like state in female mice, it has been widely used to study diseases associated with or affected by menopause, such as atherosclerosis (Mayer, Dyer et al. 2005), insulin resistance (Romero-Aleshire, Diamond-Stanic et al. 2009), neuroprotection following stroke (Schauwecker, Wood et

al. 2009), and osteoporosis (Wright, L. E., Christian et al. 2008). Given that VCD treatment is sufficient to induce menopause-like hormonal changes while leaving the ovary intact, it is an invaluable tool for studying the effects of menopause on ovarian tumour progression.

In vivo models of ovarian cancer

Until recently there were few *in vivo* models of EOC; therefore most research centered on clinical studies or *in vitro* models which cannot fully replicate all of the interactions present in living organisms. Most *in vivo* models that are available suffer the limitations of low disease penetrance, long tumour latency, tumours that did not develop *in situ* and/or ovarian histology that was not representative of the majority of EOCs in women (Vanderhyden, Shaw et al. 2003; Garson, Shaw et al. 2005).

The most widely studied non-transgenic animal models of ovarian cancer include the laying hen model and mouse xenograft models. Hens that are maintained under egg-laying regimens for at least two years develop sporadic ovarian adenocarcinomas, but with a low incidence of 9-39% of hens studied, depending on their age (Fredrickson 1987). Despite the low incidence of ovarian tumours, the laying hen model of ovarian cancer has been widely used to study disease progression including tumour histology, (Barua, Bitterman et al. 2009) vasculature (Urick, Giles et al. 2008; Barua, Bitterman et al. 2010), antitumour immunity (Barua, Edassery et al. 2009), biomarkers (Jackson, Anderson et al. 2007) and potential disease modulators such as flaxseed (Ansenberger, Richards et al. 2010), aspirin (Urick, Giles et al. 2009), E-cadherin (Ansenberger, Zhuge et al. 2009), and progestins (Barnes, Berry et al. 2002). The limitations to this model are

the low penetrance of EOCs, the long latency, and the variability between animals in disease initiation and progression, making it difficult to predict and study disease onset and progression. Spontaneous ovarian tumours have been reported in other animals, such as turkeys (Moore and Siopes 2004), rats (Sharrow, Ronnett et al. ; Gregson, Lewis et al. 1984; Walsh and Poteracki 1994), and mice (Beamer, Hoppe et al. 1985; Tillmann, Kamino et al. 2000), but are generally rare events that do not recapitulate human epithelial ovarian cancer histology and progression.

Human ovarian cancer cells xenografted into immunodeficient mice are commonly used to study ovarian cancer progression and to test novel therapeutics (Vanderhyden, Shaw et al. 2003; Shaw, Senterman et al. 2004). Since cell lines used in xenograft studies are derived from human ovarian cancers, xenograft models cannot be used to examine the early events involved in tumourigenesis, such as the cells of origin, preneoplastic lesions, and early genetic events involved in transformation and invasion. Another limitation to using xenograft models is the deficiencies in their immune systems, since tumourigenesis in humans occurs in the presence of the immune system. As such, more relevant animal models of *in situ* ovarian cancer are required.

Transgenic mouse models of cancer

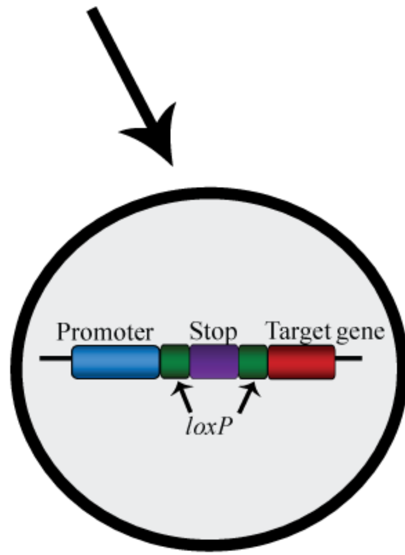
The first generation of genetically modified mouse models of cancer sought to either knock out expression of tumour suppressor genes or constitutively activate oncogenes, either in specific tissues or throughout the entire organism (Jonkers and Berns 2002). In order to do this, the first transgenic mouse models of cancer involved injecting

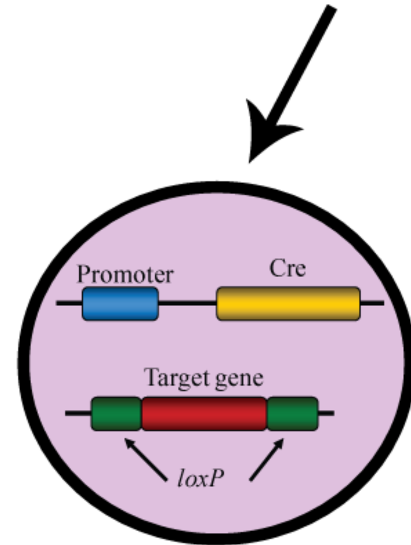
DNA copies of known oncogenes, such as large and small T antigens (SV40 TAg) and Myc, into mouse oocytes, generating mice in which tumours developed (Brinster, Chen et al. 1984; Stewart, T. A., Pattengale et al. 1984). The capacity to grow mouse embryonic stem cells and to specifically modify the genome via homologous recombination was considered a refinement for generating transgenic mice because of the ability to site direct specific mutations or DNA (Robertson, Bradley et al. 1986; Thomas and Capecchi 1987). A limitation to this technology was the occasional embryonic lethality of some germline genetic manipulations which eventually motivated the development of conditional and tissue specific mutations in transgenic mouse models (Jonkers and Berns 2002).

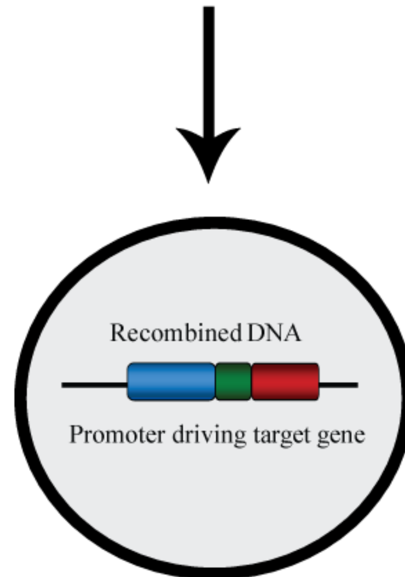
The ability to temporally and spatially control the expression of specific genes and mutations allowed for more accurate modelling of *in situ* tumour formation (Jonkers and Berns 2002). This was achieved with the development of the FLP/*FRT* and Cre/*loxP* systems and the retroviral receptor TVA system (Sauer 1998; Fisher, Orsulic et al. 1999; Jonkers and Berns 2002). Site directed homologous recombination most commonly utilizes the Cre/*loxP* system and can be achieved by somatic delivery of Cre recombinase (for example, via engineered adenoviruses containing *Cre* DNA) or by intercrosses with *Cre* transgenic mice that employ tissue specific promoters to drive *Cre* expression (Jonkers and Berns 2002). Cre recombinase can be used either to conditionally inactivate tumour suppressor genes which are flanked by loxP sites (floxed genes) or activate oncogenes that are repressed by upstream *loxP*-flanked (floxed) stop sequences (Figure 3).

Adenovirus expressing Cre

Transgenics expressing Cre







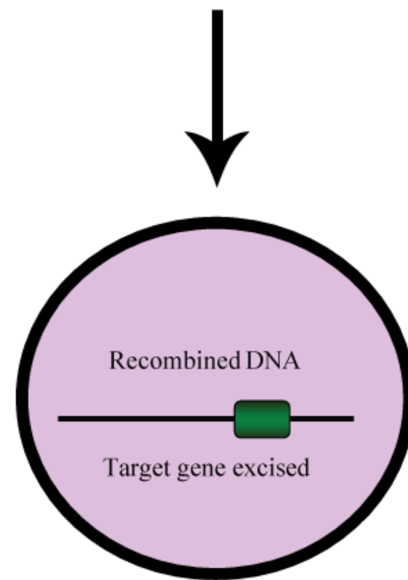


Figure 3. Schematic of the Cre/*loxP* system. Cre recombinase can be introduced to cells either by somatic delivery (ie., via adenoviruses that have Cre expression) or by generating transgenic mice with tissue specific promoters driving Cre. The Cre/*loxP* system can be used to inducibly express oncogenes or to remove tumour suppressor genes that are flanked by *loxP* sites.

Transgenic mouse models of ovarian cancer

The premium transgenic mouse models of cancer are those that have cells of origin and genetic changes consistent with the disease in humans, mimic all stages of progression, and respond similarly to chemopreventives and therapeutics. Until recently there were no transgenic mouse models of epithelial ovarian cancer available because there is no known OSE cell specific promoter. In order to circumvent this problem, mice were engineered to upregulate oncogenes or downregulate tumour suppressor genes by utilizing gene promoters that were known to be expressed throughout the reproductive tract epithelium, including the OSE cells. Eventually the Cre/*LoxP* system was also employed in the development of a new generation of conditional mouse models of ovarian cancer. Tumour suppressor genes flanked by *loxP* sites were inactivated in mice following the introduction of Cre recombinase to the somatic OSE cells directly via injection of adenoviruses expressing *Cre* under the bursal membrane that surrounds the ovary.

The first engineered mouse model of ovarian cancer, tgMISIIR-TAg mice, was achieved by utilizing the Mullerian inhibitory substance type II receptor (*MISIIR)* gene promoter to drive expression of the SV40 TAg oncogene in the mouse ovary (Connolly, Bao et al. 2003). SV40 TAg transforms cells by binding and inactivating the tumour suppressor proteins Rb and p53 (DeCaprio, Ludlow et al. 1988; Conzen and Cole 1995). Although SV40 TAg itself is not involved in the initiation of human ovarian cancer, its targets Rb and p53 are inactivated or mutated in a high percentage of cases. Loss of heterozygosity at the site of the *Rb* gene is frequently observed in ovarian cancers (between 24% and 61% of cases) and approximately 50% of advanced stage ovarian

cancers have mutations in the *TP53* gene (Liu, Heyman et al. 1994; Gras, Pons et al. 2001; Schuijer and Berns 2003).

Approximately 50% of the female tgMISIIR-TAg mice developed bilateral, poorly differentiated ovarian carcinomas within 6-13 weeks of age (Connolly, Bao et al. 2003). Immunohistochemistry showed the ovarian tumours to be positive for cytokeratins 8 and 19 and negative for inhibin α, further suggesting that the tumours arose from the OSE cells, despite the lack of specificity of the MISIIR promoter (Connolly, Bao et al. 2003). Moreover, disease progression was similar to human ovarian carcinoma, with peritoneal ascites and metastasis to the omentum and mesentery (Connolly, Bao et al. 2003).

Since the *MISIIR* promoter is not specific to the OSE cells, the Cre/*LoxP* system was utilized to specifically target the OSE cells. Double transgenic mice were created bearing homozygous floxed copies of the tumour suppressor genes *p53* and *Rb* (Flesken-Nikitin, Choi et al. 2003). Concurrent inactivation of both p53 and Rb was mediated through the intrabursal delivery of adenovirus expressing Cre recombinase (AdCre, Figure 4) (Flesken-Nikitin, Choi et al. 2003). Ovarian tumours developed in 33 out of 34 mice that were treated with AdCre and the cancer was fatal in the transgenic mice at a median age of 227 days post-AdCre injection (Flesken-Nikitin, Choi et al. 2003). These tumours had various histological features of EOC, including areas of papillae, cysts, and poorly differentiated lesions, but did not fully recapitulate any of the subtypes of EOC (Flesken-Nikitin, Choi et al. 2003). Interestingly, only 13% of the floxed *p53* and 3% of the floxed *Rb1* mice developed ovarian tumours following intrabursal AdCre injection

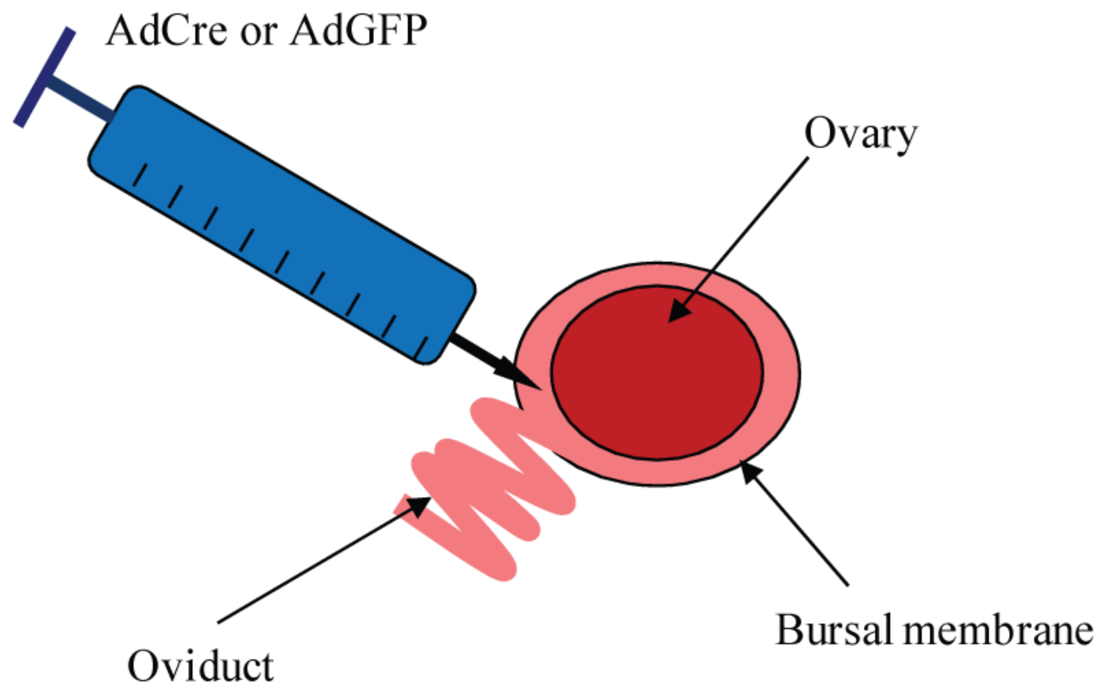


Figure 4. Schematic of the intrabursal injection procedure. Adenoviruses expressing Cre recombinase (AdCre) or eGFP (AdGFP) are injected into the bursal space near the oviduct-bursa junction.

suggesting that concomitant inactivation of at least two tumour suppressor genes is required for a high penetrance of ovarian tumours (Flesken-Nikitin, Choi et al. 2003).

Similarly, Dinulescu and colleagues activated oncogenic *K-ras* either alone or in combination with inactivation of the *Pten* tumour suppressor gene via the intrabursal injection of AdCre (Dinulescu, Ince et al. 2005). Targeting either *K-ras* or *Pten* resulted in preneoplastic ovarian lesions and endometriosis, but the combination of the two mutations was required to give rise to endometrioid ovarian carcinomas, which developed between 7-12 weeks after intrabursal injection (Dinulescu, Ince et al. 2005). A second model of endometrioid ovarian carcinoma, designed to better mimic the genetic changes that occur in women with the disease, was subsequently developed. These mice underwent conditional inactivation of the floxed *Pten* and *Apc* tumour suppressor genes, via intrabursal delivery of AdCre (Wu, Hendrix-Lucas et al. 2007). Simultaneous inactivation of both tumour suppressor genes resulted in endometrioid ovarian tumours, hemorrhagic ascites and peritoneal dissemination, similar to human disease (Wu, Hendrix-Lucas et al. 2007). It was shown in samples of human endometrioid ovarian cancers in conjunction with the $Pten^{loxP/loxP}/Apc^{loxP/loxP}$ mouse model that deregulated signalling in the PI3K/Pten and canonical Wnt pathways cooperate in the development of this specific histological subtype of EOC (Wu, Hendrix-Lucas et al. 2007). These previously developed transgenic mouse models of ovarian cancer highlight the importance of reproducing the genetic events, the tissue of origin, and the timing of onset of tumourigenesis to derive models that fully represent human disease.

Although all of these models result in ovarian tumours, they have some limitations which prevent them from fully recapitulating disease progression in humans,

either because they arise from tissues other than the OSE, result in only one histological subtype or because tumourigenesis occurs embryonically. Moreover, these models have rarely been used to study risk factors for ovarian cancer, develop biomarkers or test novel therapeutics. Most importantly, the effects of E2, P4, LH, and FSH on ovarian cancer progression have not been examined using transgenic mouse models. The goal of my PhD research project was to investigate the role of the reproductive hormones on ovarian cancer etiology using a novel transgenic model that accurately depicts ovarian cancer progression in humans.

Hypotheses

The hypotheses of this thesis are:

1) that exogenous 17β-estradiol renders the OSE cells more susceptible to malignant transformation, resulting in a higher incidence of preneoplastic lesions in the OSE of normal mice (not predisposed to ovarian cancer) and an earlier onset of disease in mice predisposed to developing ovarian cancer.

2) that 17β-estradiol will act as a mitogen in ovarian cancer and accelerate disease progression in a mouse model of ovarian cancer.

3) that exogenous progesterone will delay the onset and progression of ovarian cancer in a mouse model of the disease.

4) that combining a mouse model of ovarian cancer with the VCD mouse model of menopause will accelerate cancer progression because of the low endogenous steroid hormone levels in combination with high endogenous gonadotropin levels.

Objectives

1) To characterize the newly generated tgCAG-LS-TAg mouse model of ovarian cancer and use the mice to determine the impact of prolonged exposure to exogenous E2 and P4 on overall survival, phenotype and pathology of ovarian cancer.

This objective is addressed in Chapter 2 of this thesis. The role of the reproductive steroid hormones in ovarian cancer initiation and progression was carefully assessed in a novel transgenic mouse model of ovarian cancer developed in our lab.

2) To determine the mechanisms through which E2 is able to accelerate the progression of ovarian tumourigenesis.

Chapter 3 contains the experiments which examine the *in vitro* effects of E2 on mouse ovarian cancer ascites cell growth. This chapter also investigates the *in vivo* consequences of exogenous E2 on disease progression and identifies several gene targets which may mediate this process.

3) To determine the effect of menopause on the morphology of mouse ovaries and pathology of ovarian cancer.

The mouse model of ovarian cancer was combined with a mouse model of menopause to determine the effects of elevated serum levels of LH and FSH, in combination with ovarian germ cell depletion and reduced E2 and P4, on disease progression. A second model of menopause, the OVX mouse, was also used to examine the role of altered hormone levels in the absence of residual ovarian tissue on ovarian cancer progression. These analyses are contained in Chapter 4.

Significance

Having animal models to recapitulate the features of disease progression is crucial for understanding the etiology of ovarian cancer, for developing biomarkers and methods for early detection, and for pre-clinical testing of novel therapeutics. During this PhD project, I have characterized a novel mouse model of ovarian cancer where tumours arise from the OSE cells with sufficient latency to study the molecular and biological events involved in neoplastic transition. Since the early events in ovarian cancer are still not well understood, I expect that this model will be extremely useful for studying ovarian cancer initiation.

Women currently have the option of using combined oral contraceptives throughout their reproductive lifetime and HRT after menopause, both of which consist of estrogens and progestins, but that have opposing effects on ovarian cancer risk. Menopause (elevated LH and FSH and germ cell depleted ovaries) also affects ovarian cancer incidence and prognosis. Although epidemiological studies have indicated an association between the female reproductive hormones and the pathophysiology of ovarian cancer, the direct effects of E2, P4, LH and FSH on disease initiation and progression have not been studied using *in situ* models of the disease that might explain more directly their mechanisms of action.

The aim of this research project was to utilize a mouse model of ovarian cancer to determine the role of the reproductive hormones on ovarian cancer risk and progression. Using the tgCAG-LS-TAg mouse model, the individual actions of the steroid hormones E2 and P4 on disease progression were examined. E2 resulted in an earlier onset of

ovarian tumourigenesis, accelerated tumour progression *in vivo,* and decreased the overall survival time, which may be mediated by elevated levels of Greb1 and Stanniocalcin 2. P4 did not affect disease progression in this model. In order to examine the role of elevated LH and FSH, the tgCAG-LS-TAg mouse model of ovarian cancer was combined with the VCD mouse model of menopause, which resulted in the hormonal changes associated with menopause, slowed the progression of ovarian cancer and altered the histology of the resulting tumours to a less aggressive subtype of ovarian cancer. A better understanding of the mechanisms through which reproductive hormones alter disease progression in this mouse model will hopefully translate into improved prevention and treatment options for women at risk for developing ovarian cancer.

Chapter 2: 17β-estradiol accelerates tumour onset and decreases survival in a transgenic mouse model of ovarian cancer

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Abstract

Epithelial ovarian cancer is thought to be derived from the ovarian surface epithelium (OSE), but often goes undetected in the early stages and as a result, the factors that contribute to its initiation and progression remain poorly understood. Epidemiologic studies have suggested that the female steroid hormones are involved in ovarian carcinogenesis and that women who use hormone replacement therapy (HRT) are at increased risk of developing the disease. A novel transgenic mouse model of ovarian cancer (tgCAG-LS-TAg) was developed to examine the role of the female reproductive steroid hormones (17β-estradiol (E_2) and progesterone (P_4)) on the initiation, progression, and pathology of ovarian cancer. The mouse model utilizes the Cre/*loxP* system to induce expression of the SV40 large and small T-Antigens (SV40 TAg). Following targeted induction of the oncogene in the OSE, mice develop poorly-differentiated ovarian tumours, tumour dissemination to tissues within the abdominal cavity, and a subset develop hemorrhagic ascites. Treatment with P_4 had no impact on the disease, but E_2 altered the pathophysiology, resulting in an earlier onset of tumours, decreased overall survival time, and a distinctive papillary histology. Normal ovaries collected from mice treated with E_2, but lacking expression of SV40 TAg, displayed an increase in the areas of columnar and hyperplastic OSE cells compared to placebo treated controls. A better understanding of the mechanisms by which E_2 alters the morphology of normal OSE cells and reduces survival in this mouse model may translate into improved prevention and treatment options for women using HRT.

Introduction

The etiology of epithelial ovarian cancer is poorly understood because there are no suitable markers for its early detection. Consequently, the disease often goes undiagnosed until its late stages and it remains the most lethal of the gynecological malignancies. The ovary is surrounded by a single layer of OSE cells that normally display differing morphologies from simple squamous to cuboidal to low pseudostratified columnar. These cells are believed to undergo hyperplasia, leading to the formation of putative preneoplastic lesions such as invaginations and inclusion cysts, which eventually give rise to ovarian tumours (Auersperg, Wong et al. 2001). Once tumourigenesis is initiated in the OSE cells, they can differentiate into several histological subtypes of ovarian cancer including serous, mucinous, endometrioid, clear cell and transitional cell neoplasms (Auersperg, Wong et al. 2001).

The ovary is the main source of the female reproductive steroid hormones. The OSE cells express the estrogen receptors α and β (ERα, ERβ) and the progesterone receptors A and B (PRA, PRB) (Lau, K. M., Mok et al. 1999; Lindgren, Cajander et al. 2004). Interestingly, very little is known about the role of E_2 and P_4 in ovarian tumourigenesis, however, epidemiological studies have reported altered ovarian cancer risk associated with the use of steroid hormones. Studies have shown that oral contraceptive use (combinations of estrogens and progestins) reduces the risk of developing ovarian cancer by up to 40% and that this protection is long-lasting and may persist for 15 years or more (Beral, Hannaford et al. 1988; Ness, Grisso et al. 2000; Deligeoroglou, Michailidis et al. 2003). In contrast, hormone replacement therapy (HRT) increases the risk of developing ovarian cancer and this effect is primarily related the use

of estrogen-only HRT (Lacey, Mink et al. 2002; Glud, Kjaer et al. 2004; Lacey, Brinton et al. 2006; Beral, Bull et al. 2007; Rossing, Cushing-Haugen et al. 2007).

Previous *in vivo* and *in vitro* studies have been performed to investigate the direct effects of steroid hormones on the ovary. Bai *et al.* (Bai, Oliveros-Saunders et al. 2000) investigated the effects of prolonged E_2 exposure on the morphology of rabbit ovaries *in vivo* and found an increase in both OSE cell proliferation and the number of papillae covering the ovarian surface, but no ovarian tumours. Estrogens can also increase the proliferation of OSE cells *in vitro*, which may be due to activation of the interleukin 6 (IL-6)/STAT3 (signal transducer and activator of transcription-3) signalling pathway (Bai, Oliveros-Saunders et al. 2000; Syed, Ulinski et al. 2002). P_4, on the other hand, reduces the proliferation of OSE cells when present at high concentrations (Syed, Ulinski et al. 2001; Blumenthal, Kardosh et al. 2003). The action of P_4 appears to be mediated through a reduction of cyclin-dependent kinase (CDK) activity (specifically cyclin A and cyclin B) (Blumenthal, Kardosh et al. 2003). Interestingly, an *in vivo* study by Rodriguez *et al.* (Rodriguez, Walmer et al. 1998) demonstrated an increase in the amount of apoptosis in the OSE cells of macaques following treatment with progestin. These studies corroborate the results obtained from epidemiological studies, suggesting that estrogens may increase the risk of developing ovarian cancer by enhancing cell proliferation, whereas P_4 may offer some protection against malignant transformation of OSE cells.

Several studies have shown that mouse models of cancer behave similarly to human cancers and can respond to hormone therapy *in vivo*. Yoshidome *et al.* (Yoshidome, Shibata et al. 2000) showed that treatment with exogenous E_2 was able to

decrease the survival period, and increase tumour multiplicity and volume in a SV40 TAg mouse model of breast cancer. A small number of mouse models of ovarian cancer have been developed (Connolly, Bao et al. 2003; Flesken-Nikitin, Choi et al. 2003; Dinulescu, Ince et al. 2005; Wu, Hendrix-Lucas et al. 2007), however none have been used to study the effects of E_2 or P_4 on the disease *in vivo*. In addition, some of these models suffer the limitation of very rapid tumour onset, which limits their usefulness for studying early tumourigenesis and potential modulators of ovarian carcinogenesis. Therefore, we were interested in creating a model in which the initiation of tumourigenesis could be controlled both spatially (in the OSE) and temporally, with enough latency to determine the impact of putative disease modulators.

In the present study, we examined the ability of the reproductive steroid hormones to alter the etiology of ovarian cancer in an inducible transgenic mouse model of the disease. We conditionally activated an oncogene, SV40 TAg, in the OSE cells and treated the mice with exogenous E_2 or P_4. The tgCAG-LS-TAg mice developed primary ovarian tumours, metastasis to tissues within the peritoneal cavity, and hemorrhagic ascites. P_4 did not affect the progression of the disease in this mouse model. E_2 treatment resulted in an earlier onset of ovarian tumours, a significantly decreased survival time and altered tumour histology. Moreover, there was an increase in putative preneoplastic lesions, specifically areas of columnar and hyperplastic cells, in the OSE from mice treated with E_2. These results highlight the importance of E_2 on the initiation of ovarian cancer and provide a model for studying the mechanisms by which E_2 modulates the progression of this disease.

Materials and Methods

Generation of tgCAG-LS-TAg Transgenic Mice

All animal experiments described in this study were performed according to the *Guidelines for the Care and Use of Animals* established by the Canadian Council on Animal Care. The pCAG-LS-TAG plasmid was constructed by subcloning the Sal1 fragment, containing the early region of SV40 TAg, from plasmid pUBC-TAg into the unique Xho1 site of pCALL2-IRES-EGFP (a gift from Dr. Corrine Lobe, University of Toronto, Toronto, ON, Canada). The pCAG-LS-TAg plasmid expresses the β-geo gene (a fusion of β-galactosidase (β-gal) and neomycin phosphotransferase genes) from the CMV early enhancer/chicken β-actin (CAG) promoter. The β-geo gene and three downstream polyA signals (3xpolyA) are flanked by *loxP* sites. Exposure to Cre recombinase results in the deletion of the β-geo/3xpolyA cassette leading to the juxtaposition of the TAg immediately downstream of the CAG promoter to allow its efficient expression. The plasmid pCAG-LS-TAg was digested with Xmn1 and the DNA fragments bearing the promoter/gene sequences were separated and purified by agarose gel electrophoresis. Purified DNA of the transgene was microinjected into the male pronuclei of FVB/n single cell embryos to yield transgenic founder mice (Garson, Macdonald et al. 2003). Founder mice and subsequent generations were identified by PCR analysis of genomic DNA using primers and conditions as previously described (Garson, Macdonald et al. 2003).

Adenovirus and Hormone Administration

Recombinant adenoviruses Ad5CMVeGFP (AdGFP) or Ad5CMVCre (AdCre) (Vector Development Laboratory, Houston, TX, USA) were delivered to the OSE *in vivo* via intrabursal injection, as previously described (Clark-Knowles, Garson et al. 2007). Briefly, six to eight week old tgCAG-LS-TAg virgin females were anesthetized via intraperitoneal injection of Avertin (2.5% v/v in 0.85% NaCl, 0.015–0.017 ml/g body weight; Sigma-Aldrich). Approximately 10μl of adenovirus (4.0×10^7 pfu/μl in PBS) was delivered to both the left and right ovaries in each animal. A 60-day slow release hormone pellet (Innovative Research of America, Sarasota, FL) was surgically implanted (subcutaneously in the neck region) at the same time as the intrabursal injection. Mice were treated with either E_2 (0.25 mg/pellet), P_4 (50 mg/pellet), or a placebo pellet. Mice were housed on a 12L:12D cycle, with free access to food and water. In order to examine the early lesions on the ovaries, a subset of animals were euthanized at early time points (day 65, 80 and 90 post intrabursal injection), before they reached a loss-of-wellness endpoint. The survival time reflects the amount of time from the day of exposure to Cre recombinase until the day when the mice reach a loss-of-wellness endpoint including: weight loss > 15 % of body weight, body weight increase > 5 g with respect to age-matched, non-tumourigenic controls, presence of abdominal distension that impairs mobility or affects wellness, respiratory distress, dehydration, anorexia, and/or diarrhea. All animals were euthanized via CO_2 asphyxiation.

Preparation and Analysis of Tissues

Tissues were collected from mice at necropsy, and the volume of ascites and wet weight of the total tumour burden were recorded. Tissues were fixed in 10% neutral-buffered formalin overnight, transferred to 70% ethanol, and paraffin-embedded. For histological analyses, 3-5 μm sections were cut and stained with hemotoxylin and eosin (H&E). Tissue sections were scanned using the Aperio ScanScope and images were captured using the Aperio ImageScope program (Aperio Technologies, Inc., Vista, CA, USA).

Immunohistochemical Analysis of Tissues

Immunohistochemistry (IHC) was performed using the following antigens: SV40 TAg (1:100 antibody dilution, Santa Cruz); pan-CK (pre-diluted, Abcam); inhibin-α (1:25, DAKO); synaptophysin (1:100, BD Biosciences); CD45R (1:100, Abcam); calretinin (pre-diluted, Zymed); PLAP (1:100, Abcam); S100α (1:500, Novus Biologicals); CK19 (Troma-3, 1:50, Developmental Studies Hybridoma Bank); ERα (1:50, Santa Cruz); PR (1:50, LabVision), and Ki67 (1:25, DAKO). Paraffin sections obtained from at least four mouse tumours per treatment group were deparaffinized in xylene and rehydrated in a graded series of alcohols according to standard protocol. Antigen retrieval was conducted in citrate buffer following standard procedures. Endogenous peroxidase activity was blocked by incubating the slides in 3% hydrogen peroxidase for 10 min. Serum free protein block and background reducing antibody diluent (DAKO) were used according to the manufacturer's instructions. Tissue sections were incubated overnight with the primary antibody (at room temperature in a humidified

chamber) and then rinsed with Stockholm PBS. Developing was performed with diaminobenzidine (DAB) as a substrate (0.2% DAB, 0.001% H_2O_2, Sigma-Aldrich). Sections incubated with PLAP, S100α, ERα or PR antibodies (rabbit antibodies) were then incubated with Rabbit Polymer HRP (DAKO) according to the manufacturer's instructions. Tissues stained with CD45R, CK19, or Ki67 (rat antibodies) were incubated with rat secondary antibodies (1:200, DAKO), followed by Streptavidin HRP (1:200, DAKO) and developed with DAB. A mouse on mouse kit (Vector) was used according to the manufacturer's instructions, to avoid cross-reactions when staining mouse tumours with mouse antibodies (SV40 TAg, pan-CK, inhibin-α, synaptophysin, calretinin). Slides were counterstained with hematoxylin, dehydrated in a series of alcohols and xylenes, and coverslipped.

Evaluation of Tumour Proliferation

Proliferation was determined by counting the number of Ki67 positive nuclei (by IHC) as a percentage of the total number of nuclei in 3 random fields of view (1000x magnification) which were averaged to obtain a mean for the tumour. Four tumours per group (AdCre + placebo, AdCre + E_2 and AdCre + P_4) were stained and counted. Quantitative RT-PCR was used to determine the amount of Cyclin D1 mRNA expression in the tumours. RNA was collected from 3 tumours per group using the RNeasy Kit (Qiagen) and cDNA was prepared using the Superscript III First-Strand Synthesis System for RT-PCR kit (Invitrogen) according to the manufacturers' instructions. 100 ng of cDNA (per tumour) was used to determine expression of Cyclin D1 relative to mouse spleen cDNA (see Supplemental Data for primer sequences).

Measuring Proliferation and Preneoplastic Lesions in the OSE of tgCAG-LS-TAg Mice

tgCAG-LS-TAg mice underwent surgery, receiving intrabursal injection of AdGFP and either a placebo or E_2 pellet (as described above). Mice were euthanized 30, 45 or 60 days following intrabursal injection (n=3 mice/group) while the hormone pellets were still active. Ovaries were fixed in formalin and sectioned (3-5µm). 9-12 ovaries per treatment (AdGFP + placebo vs. AdGFP + E_2) were randomly selected and stained by IHC for expression of Ki67. The total number of Ki67 positive OSE cells was counted. The linear distance (in µm) of columnar cells and areas of hyperplasia was measured and expressed as a percentage of the entire linear distance (perimeter) of the OSE layer using the Aperio ImageScope software. For most ovaries, the Ki67 IHC was performed twice and the two measurements per ovary were averaged.

Statistical analyses

All data were plotted using GraphPad Prism software (version 3.02: GraphPad Software, San Diego, CA, USA). Kaplan-Meier survival curves were compared using a Logrank test. tgCAG-LS-TAg tumour weights, ascites volumes, and Ki67 and cyclin D1 expression were compared using one-way ANOVA tests, and *t* tests were used to compare the number of proliferating cells (Ki67 expression), areas of columnar cells and areas of hyperplasia in the OSE. Significance was inferred at $P < 0.05$.

Results

Generation of the tgCAG-LS-TAg inducible mouse model of ovarian cancer.

A new line of mice was generated to address the goal of creating a model in which the initiation of tumourigenesis could be confined to the OSE with enough latency to determine the impact of putative disease modulators. Following Cre-mediated excision of a lox-stop cassette, the tgCAG-LS-TAg mice express SV40 TAg under the control of the ubiquitous CAG promoter (Fig. 1A). In the absence of Cre recombinase, the tgCAG-LS-TAg mice express β-gal. Ten founders which transmitted the transgene through the germline were screened for β-gal expression in the ovary. Two lines showed reproducible expression of β-gal in the OSE (Fig. 1B). Of these two lines, only one was found to express SV40 TAg in the OSE one week after the intrabursal administration of adenovirus expressing Cre recombinase (AdCre). In the tgCAG-LS-TAg line, the expression of β-gal was not uniform across the OSE, but was highly localized to recent sites of ovulation (Fig. 1B), based on the presence of underlying corpora lutea. Similarly, immunohistochemical analysis of SV40 TAg expression in ovaries one week following injection of AdCre under the bursal membrane revealed TAg expression only in one or two patches of OSE in each section (data not shown).

Following the intrabursal delivery of AdCre, nine of eleven tgCAG-LS-TAg mice developed large, well-vascularized ovarian tumours with a median survival of 113 days post-injection. Primary ovarian tumours were frequently (89%) accompanied by the dissemination of malignant cells throughout the peritoneal cavity to tissues such as the diaphragm, liver, pancreas, spleen, intestines, body wall, uterus and/or oviduct, consistent

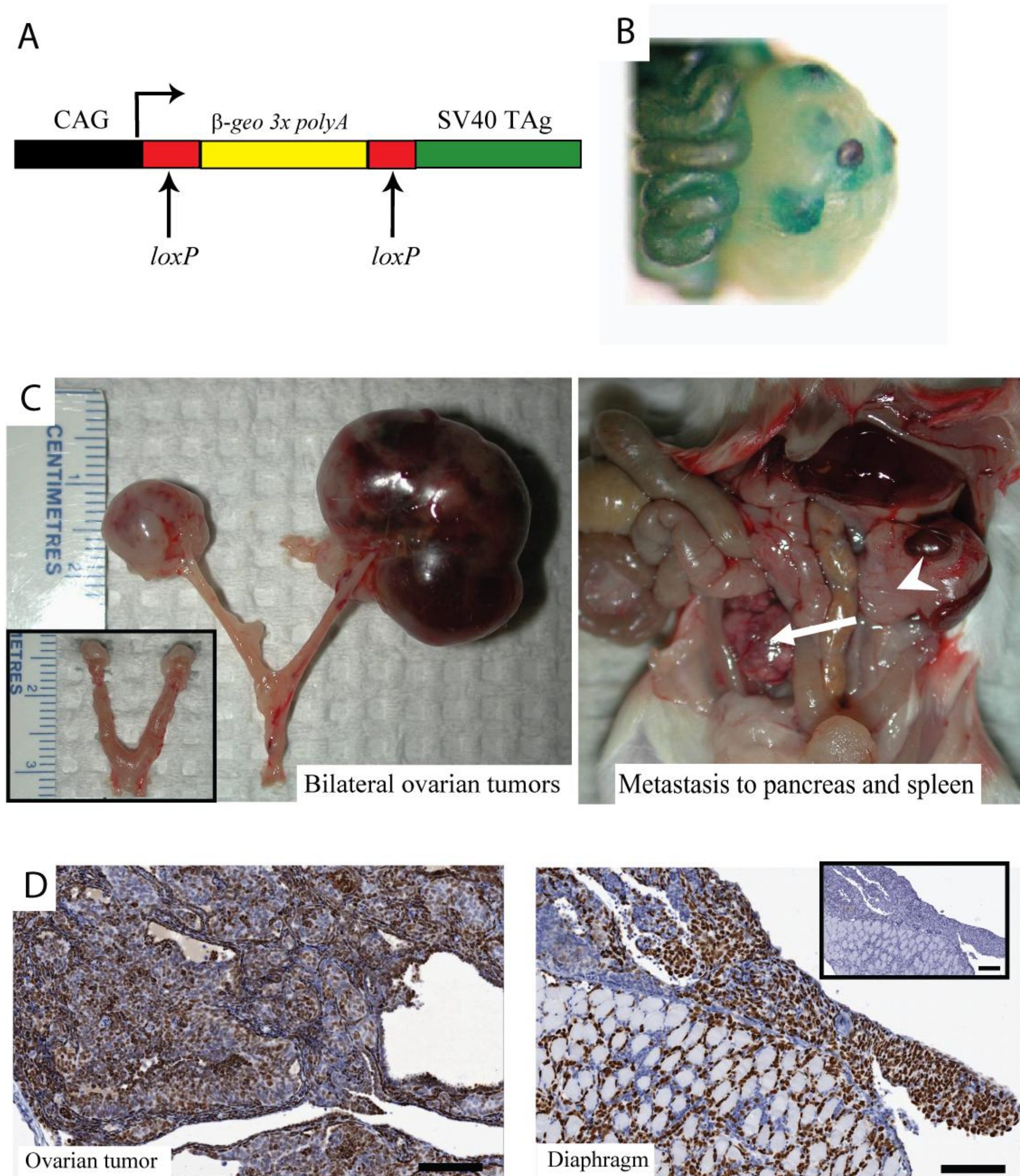


Figure 1. Generation of the tgCAG-LS-TAg transgenic mouse model of ovarian cancer. A) The tgCAG-LS-TAg construct. B) Expression of β-galactosidase in the ovary and oviduct of a tgCAG-LS-TAg mouse. C) Representative bilateral ovarian tumours and metastases from an ovarian tumour (arrow) to the spleen and pancreas (arrowhead) obtained from tgCAG-LS-TAg mice following the intrabursal injection of AdCre. Inset is a representative normal wild type mouse reproductive tract. D) IHC demonstrating the expression of SV40 TAg in the left ovarian tumour and diaphragm tumour from a tgCAG-LS-TAg mouse injected with AdCre. Inset is a no primary antibody control. Scale bars represent 100 μm. Figure 1B was previously published (Garson, Shaw et al. 2005) and is reprinted here with permission.

with human disease (Fig.1C). Given the local administration of AdCre to the ovary only, we refer to the disseminated tumours as metastasis. A proportion of the mice (64%) developed peritoneal ascites with volumes ranging from 0.5 to 7.5 ml. Of the remaining two tgCAG-LS-TAg mice that did not present with ovarian tumours at necropsy, one mouse died of tumours associated with the spleen, pancreas, liver, and diaphragm while another had an accumulation of ascites and adhesions in the abdomen.

Immunohistochemical analyses of a subset of the ovarian tumours indicated that they expressed SV40 TAg (Fig. 1D), confirming that tumourigenesis was dependent on Cre-mediated activation of the SV40 TAg transgene. PCR analysis of genomic DNA obtained from mouse ascites cells confirmed recombination of the transgene (data not shown). Moreover, the non-ovarian tumours stained positive for SV40 TAg (by IHC) and histologically resembled the primary ovarian tumours (Fig. 1D), further suggesting that these are metastases. The effects of AdCre were restricted to the tgCAG-LS-TAg mice, because ovaries collected from non-transgenic FVB/n mice injected intrabursally with AdCre (n=8) were histologically normal (data not shown). Untreated tgCAG-LS-TAg mice were monitored up to one year of age and did not develop tumours.

Latency of ovarian tumour development.

In an attempt to characterize the early events involved in ovarian tumourigenesis, tgCAG-LS-TAg mice were injected with AdCre (under the bursa) and euthanized at early time points. Expression of SV40 TAg remained localized to the OSE and bursa cells 65 days after intrabursal injection of AdCre (n=2), and at this time point the OSE was

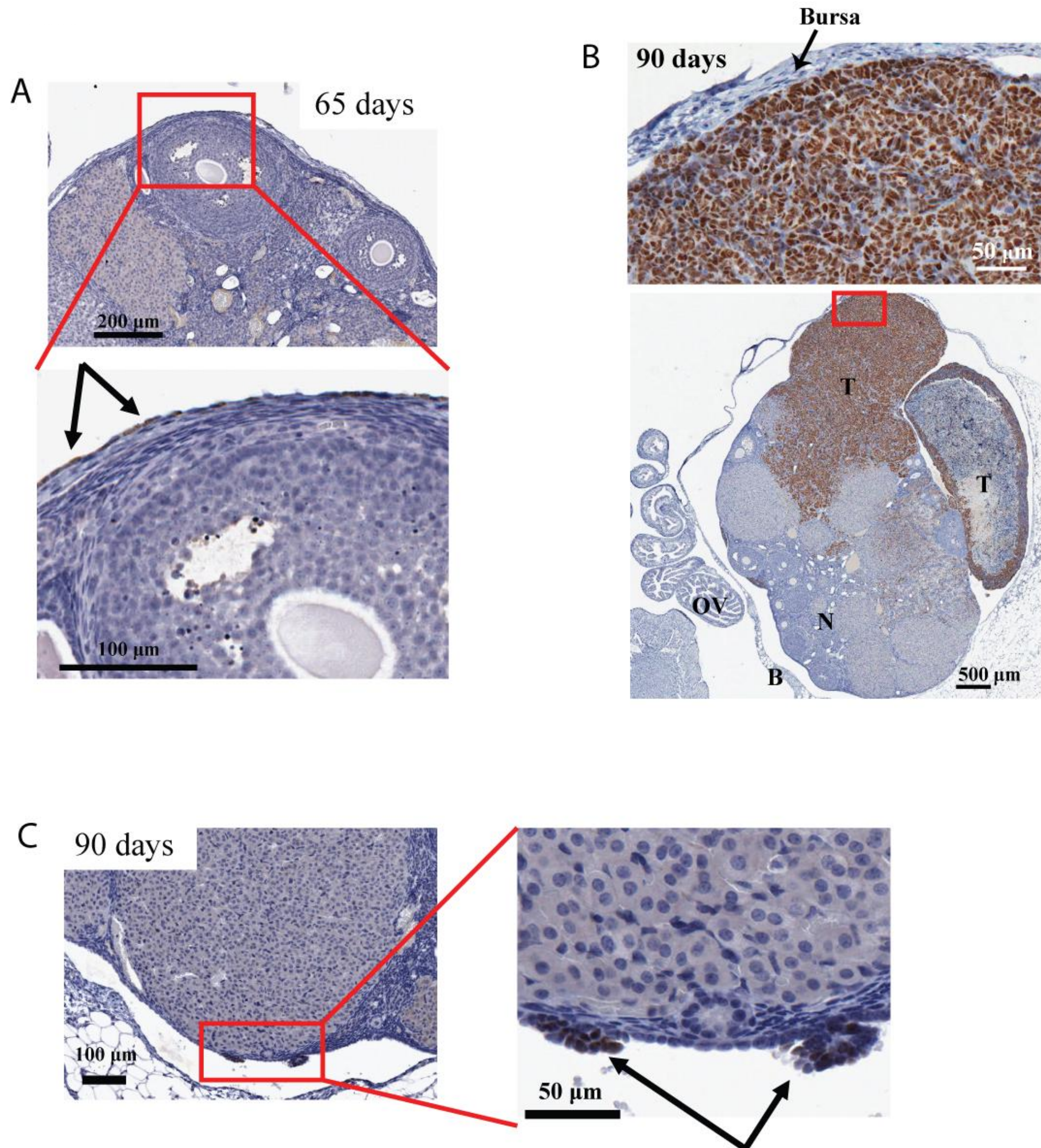


Figure 2. Immunohistological analysis of SV40 TAg expression in the OSE cells demonstrating the latency from the time of transgene induction until early tumourigenesis. A) OSE cells are morphologically normal and exhibit SV40 TAg transgene expression (arrows) 65 days after the intrabursal delivery of AdCre. B) SV40 TAg positive nascent tumour cells are observed invading into the ovary at 90 days following AdCre administration (OV – oviduct; B – bursa; N – normal ovary; T – tumour). C) Small clumps of transgene positive cells (arrows) can be seen budding off of the surface of the ovary at 90 days after injection. The ovaries in Figure 2B and 2C were collected from two separate mice.

morphologically normal, displaying only simple squamous to cuboidal cells (Fig. 2A). Other than the OSE, there were no TAg positive cells within the ovary and the stroma and follicles were histologically normal. However, 80 to 90 days after introduction of Cre recombinase (n=8), nascent tumours began to form from the OSE layer and invade into the ovary (Fig. 2B and 2C). These results suggest that there is a long latency from the time of SV40 TAg induction (between days 0-7) until the initiation of tumourigenesis (between days 80-90).

Exogenous E_2 decreases survival of tgCAG-LS-TAg mice.

To examine the effects of exogenous steroid hormones on tumour development in the transgenic model, a subcutaneous hormone pellet which released either P_4 or E_2 for 60 consecutive days was implanted coincidentally with the intrabursal administration of AdCre. To serve as controls for the intrabursal injections, exposure to adenovirus, and administration of hormones, a subset of tgCAG-LS-TAg mice were similarly injected with adenovirus expressing eGFP (AdGFP) alone (with a placebo pellet, n=9) and AdGFP with either an E_2 (n=6) or a P_4 (n=9) pellet. The AdGFP-injected mice treated with or without hormones were euthanized one year post intrabursal injection and did not display any gross signs of ovarian neoplasms at necropsy (Fig. 3). Mice treated with E_2 occasionally experienced short-term morbidity due to bladder problems. Four E_2-treated mice were euthanized for complications related to a distended bladder and were excluded from the study (data not shown). This side-effect is believed to be specific to certain strains, including FVB/n, and has been observed previously in breast and cervical cancer

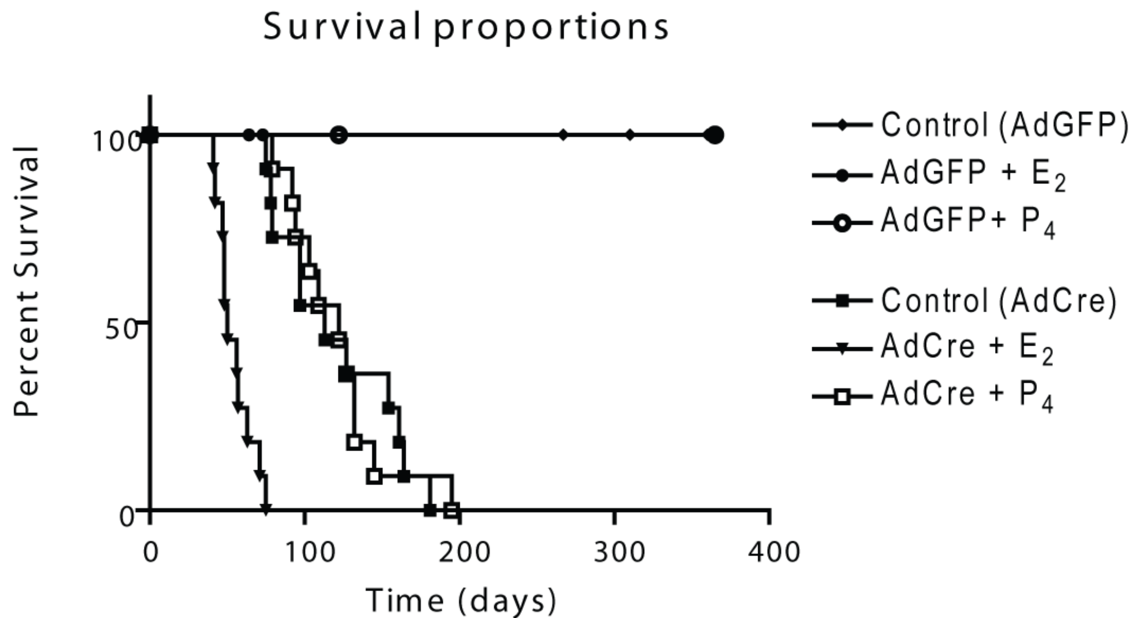


Figure 3. Kaplan-Meier survival curves demonstrating the effects of exogenous steroid hormones on the survival time of tgCAG-LS-TAg mice. The control (AdCre) mice had a median survival time of 113 days after intrabursal injection (range 75-181 days, n=11), whereas the AdCre + E_2 and AdCre + P_4 treated mice had a median survival of 50 days (range 41-75 days, n=11) and 122 days (range 79-195 days, n=11), respectively. There was a significant difference in survival times between the E_2 (+ AdCre) treated mice and the controls (AdCre) not treated with hormones (Logrank test, $P < 0.0001$). There was no significant difference in survival of mice treated with AdCre + P_4 compared to AdCre controls. The mice treated with AdGFP with or without hormones survived for 365 days without any evidence of tumours.

models (Elson, Riley et al. 2000; Shai, Pitot et al. 2008). Radioimmunoassay of serum samples confirmed that the placebo pellets do not alter E_2 or P_4 levels in the mice as compared to serum obtained from mice without pellets (data not shown). Exposure to 60 day slow release hormone pellets elevated circulating levels of hormones for two months, and then the hormone levels gradually returned to basal levels after 90 to 100 days post insertion (Supplemental Fig. 1). The serum level of E_2 was approximately 25 times higher (than basal level) while the pellet was delivering hormone. P_4 levels were approximately 7 times higher while the pellet was active (Supplemental Fig. 1).

When compared to controls receiving AdCre and no exogenous hormones, the P_4 treated mice (n=11) did not have a significantly different survival time (113 vs. 122 days post-AdCre, respectively; Fig. 3) but did display a trend towards a decreased incidence of ovarian neoplasms (64% vs. 82% in controls). Some of the P_4 treated mice that did not develop ovarian tumours, died of tumours in the liver, spleen and pancreas. In contrast, the tgCAG-LS-TAg mice injected with AdCre and treated with exogenous E_2 (n=11) had a significantly decreased survival time compared to controls. Their median survival was only 50 days post-intrabursal injection as compared to 113 days in the AdCre control mice (Fig. 3). This decrease in survival time could not be explained by additional induction of the transgene by E_2. Promoter activity of the transgene was measured in tgCAG-LS-TAg mouse OSE cells which were grown *in vitro* and treated with either E_2 or vehicle control. A LacZ assay confirmed that E_2 does not increase the amount of β-gal expression (upstream of SV40 TAg in the transgene, Fig. 1A) in the OSE cells (data not shown). Moreover, Western blot analysis indicated that treating tgCAG-LS-TAg mouse

OSE cells with E_2 *in vitro* does not alter the expression of SV40 TAg (Supplemental Fig. 2).

E_2 accelerates tumour onset and alters disease phenotype.

The shortened survival of mice treated with E_2 could be mediated by a variety of mechanisms, including acceleration of tumour onset, increased proliferative rate of tumour cells and/or modification of tumour histology to a more aggressive subtype of ovarian cancer. Acceleration of tumour onset is likely the most important factor, since E_2 treatment resulted in a substantial tumour burden that was lethal at a median of 50 days. In the control mice (AdCre + placebo) microscopic tumours were not evident until approximately 90 days after intrabursal injection (Fig. 2B, 2C), suggesting that the initiation of the disease can be accelerated with exogenous E_2. At the loss of wellness endpoint (AdCre + E_2 – median 50 days) the tumour burden in E_2 treated mice was not significantly different from control mice (AdCre + placebo – median 113 days) (Fig. 4A), although it is interesting to note that E_2 treatment decreased the incidence of peritoneal ascites accumulation (only 2 E_2 treated mice developed ascites, Fig. 4A). Despite reduced ascites, mice treated with AdCre + E_2 developed more tumours in the urogenital tissues (uterus, cervix, bladder and urethra; Supplemental Fig. 3) than control or P_4-treated mice. Interestingly, E_2 treatment did not affect the cellular proliferation rate of the ovarian tumours (Fig. 4B). Ki67 expression was similar in the tumours of mice treated with AdCre + placebo, AdCre + E_2, or AdCre + P_4. Moreover, Cyclin D1 mRNA expression was not increased by steroid hormone treatment (Fig. 4C) further confirming

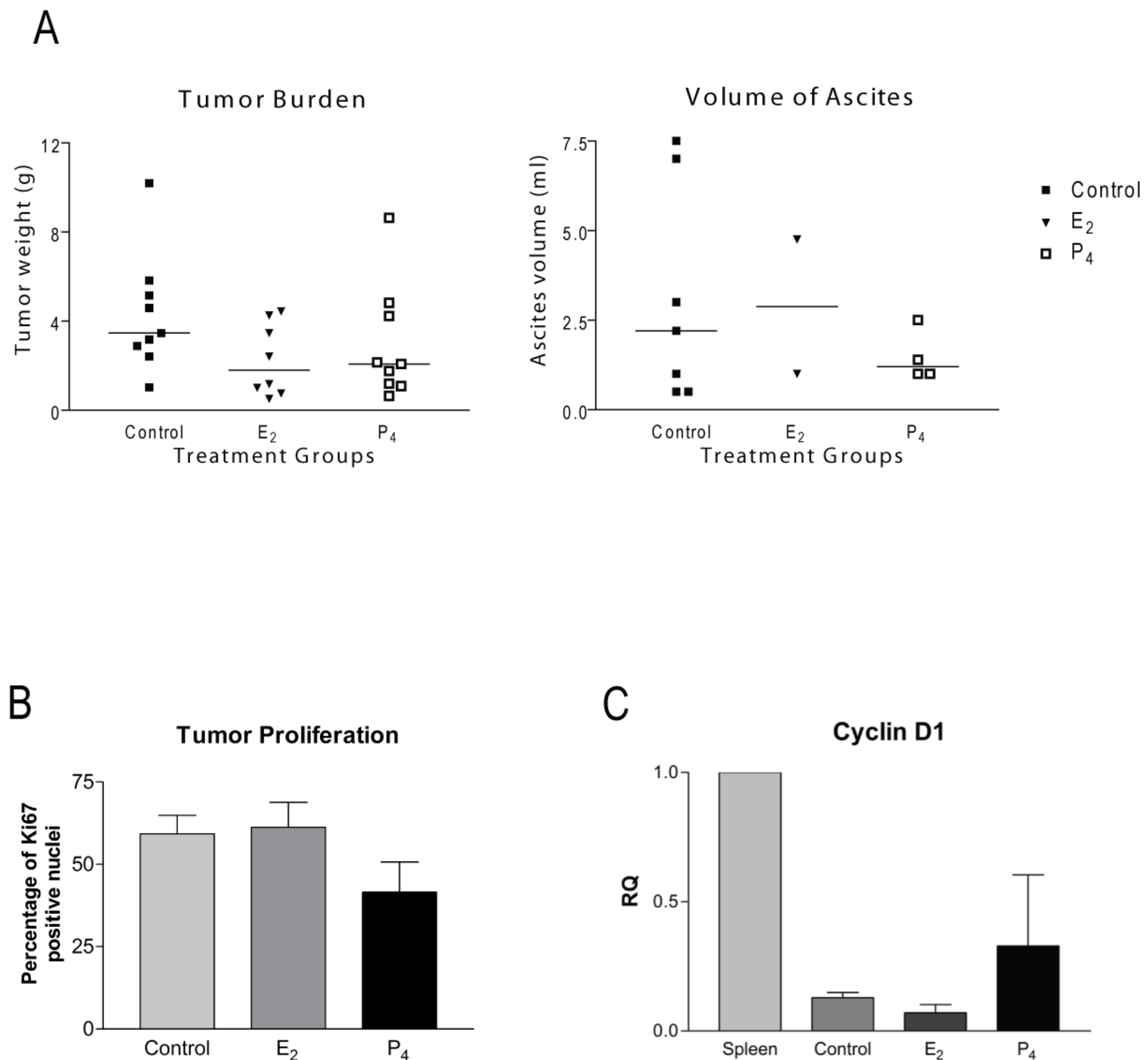


Figure 4. The effects of steroid hormones on ovarian tumour burden and proliferation. A) There were no significant differences in total tumour weight or ascites volume among mice treated with E_2, P_4 or placebo controls. Each data point represents a measurement from one mouse and the bars indicate the median for each group. B) Ki67 expression was used to determine the amount of cellular proliferation in the tgCAG-LS-TAg tumours. There were no differences in tumour proliferation between the placebo-, E_2- or P_4-treated mice. C) Similarly, there were no differences in cyclin D1 expression between the treatment groups, as measured by relative real-time quantitative PCR.

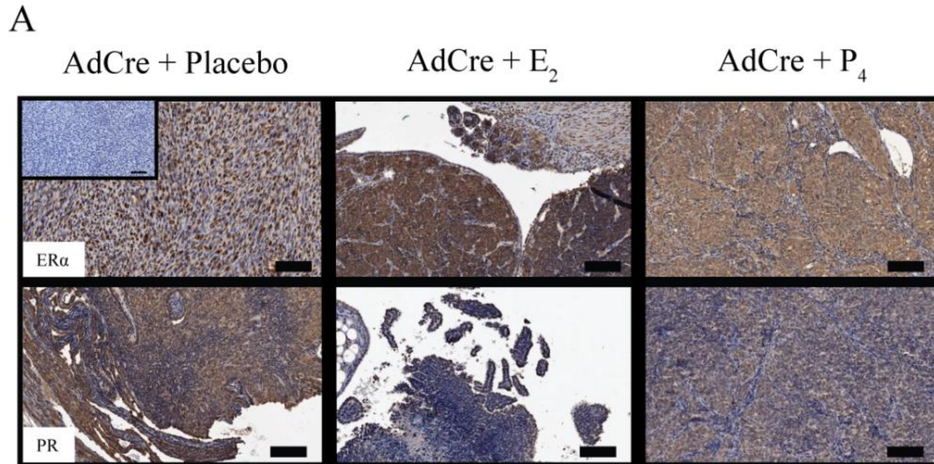
that E_2 does not act as a mitogen in the tgCAG-LS-TAg tumours.

IHC was used to help characterize the tgCAG-LS-TAg tumours (Supplemental Table 1). Tumours from all groups displayed varying levels of cytokeratins and inhibin α, suggesting that the tumours are comprised of both epithelial and stromal components (Supplemental Table 1). S100α was expressed by all groups, whereas PLAP and synaptophysin were markedly absent (Supplemental Table 1). There was a high level of expression of ERα and PR in tumours obtained from all of the treatment groups, suggesting that the effects of the hormones may be mediated through direct stimulation of the steroid receptors (Fig. 5A).

Although the IHC analysis yielded similar results for the three tumour groups, careful examination of the tumour sections revealed notable differences in their histologic appearance. The AdCre control tumours had characteristics reflective of sex cord stromal tumours, including cysts and areas of cords or insular formations (Fig. 5B). P_4 treatment resulted in a more diffuse pattern and a higher incidence of zellballen, but histologically resembled the control (AdCre + placebo) tumours (Fig. 5B). The tumours from E_2-treated mice displayed a strikingly different histology (compared to controls) with a more diffuse appearance, abundant surface papillae, and large areas of necrosis (Fig. 5B).

E_2 treatment induces putative preneoplastic lesions in the mouse OSE.

E_2 treatment of tumourigenic tgCAG-LS-TAg mice revealed that the steroid hormone decreases the latency of the disease, suggesting that E_2 may not only affect the cancer cells, but may also have direct effects on the normal OSE cells prior to



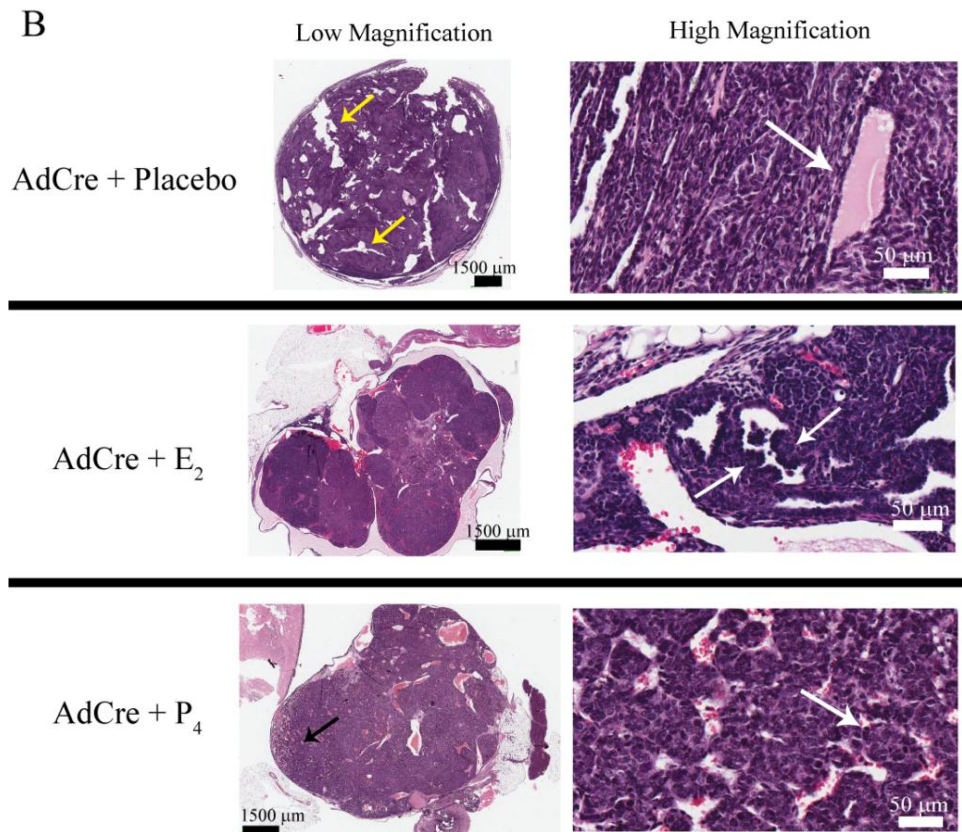


Figure 5. The histology of tgCAG-LS-TAg tumours from mice treated with placebo, E_2 or P_4. A) Representative tumour sections from an AdCre control tumour, E_2 tumour and P_4 tumour, which have similar levels of expression of ERα and PR. The inset is a no primary antibody control. Scale bars represent 100 µm. B) Histologically the tumours are of high mitotic grade and diffuse. There are glandular structures throughout the AdCre + placebo tumours (arrows). The E_2 treated mice have papillary structures at the surface and within the tumours (arrows). The tumours from P_4 treated mice display areas of zellballen (arrows).

transformation. In order to determine if E_2 affects proliferation or morphology of normal OSE cells (before transformation), mice that did not express the oncogene (tgCAG-LS-TAg mice intrabursally injected with AdGFP) were treated with either a placebo pellet or an E_2 pellet. The ovaries were collected while the hormone pellet was active (between day 30 and day 60) and Ki67 IHC was used to determine the proliferation rate of OSE cells. Interestingly, there were no differences in OSE cell proliferation between E_2 treated mice and placebo controls (Fig. 6A), but E_2 treatment did alter the morphology of the cells. The regions of the ovarian surface where OSE cells were abnormal were measured and expressed as a percentage of the entire perimeter of the ovary. E_2 treatment resulted in significantly more areas of columnar cells (Fig. 6B) and hyperplastic cells (Fig. 6C). These alterations in the OSE are believed to be preneoplastic lesions, suggesting that exogenous E_2 may decrease tumour latency due to changes in the morphology of the OSE cells which render them more susceptible to transformation.

Discussion

One of the limitations of the few currently available transgenic mouse models of ovarian cancer (Connolly, Bao et al. 2003; Flesken-Nikitin, Choi et al. 2003; Dinulescu, Ince et al. 2005; Wu, Hendrix-Lucas et al. 2007), is that they suffer rapid tumour progression which precludes characterization of the events involved in early tumourigenesis. We have developed a mouse model of ovarian cancer with sufficient tumour latency to permit the evaluation of factors believed to modulate the etiology of

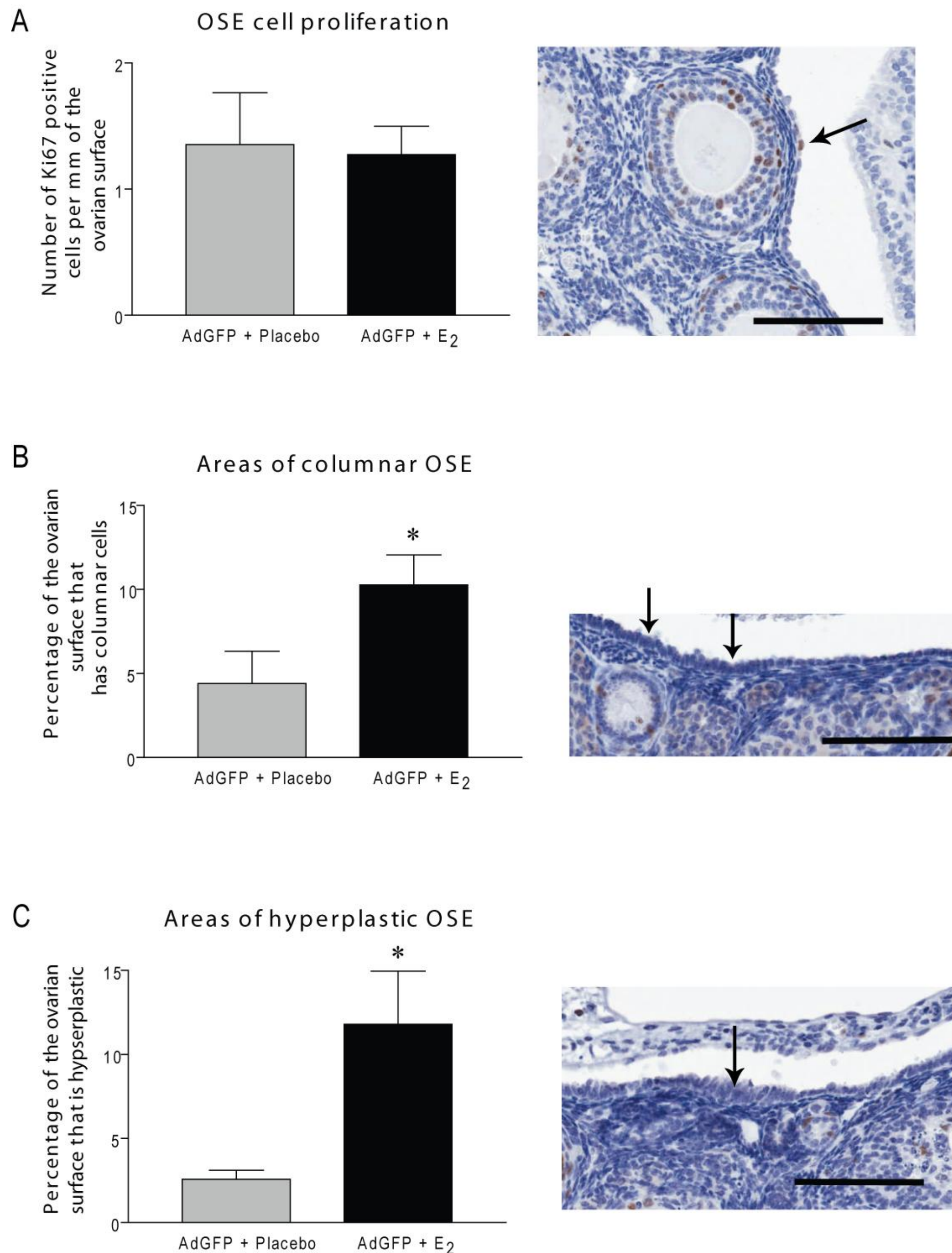


Figure 6. The proliferation and morphology of OSE cells treated with placebo or E_2. A) All of the proliferating cells (expressing Ki67, arrow) in the OSE were counted and expressed as the number of proliferating cells per millimeter of the ovarian surface. B and C) The linear distance of the areas of columnar cells (arrows) and hyperplastic cells (arrow) were measured and expressed as a percentage of the entire length of the OSE. E_2 treatment resulted in a higher percentage of columnar cells (*t* test, $P = 0.04$) (B) and hyperplastic cells (*t* test, $P = 0.02$) (C) in the surface of the ovary. Ovaries collected at three time points throughout steroid hormone exposure (day 30, 45, and 60) were combined for analysis (AdGFP + placebo, n = 9; AdGFP + E_2, n = 12). Scale bars represent 100 μm. Error bars indicate the SEM. *, Significant difference from placebo control.

the disease. Treatment of control tgCAG-LS-TAg mice (AdGFP) with exogenous steroid hormones revealed that neither E_2 nor P_4 on their own are sufficient to induce tumours. Exogenous P_4 had minimal effects on tumour progression in the tgCAG-LS-TAg mice (injected with AdCre), however, treatment with E_2 resulted in an earlier onset of ovarian tumour development, altered tumour histology and decreased overall survival time. Moreover, E_2 treatment alone altered the morphology of OSE cells, inducing putative preneoplastic lesions in the OSE even in the absence of the SV40 TAg oncogene. This model may be useful for elucidating the mechanisms by which exogenous estrogens increase ovarian cancer risk in women.

The tgCAG-LS-TAg model may also be useful for studying the biology of ovarian cancer progression, since a large proportion of the mice develop ascites and dissemination of tumours throughout the peritoneal cavity, which are both characteristics of disease progression in women (Naora and Montell 2005). Common locations for tumour dissemination in this model included the peritoneal membrane, diaphragm, mesentery of the intestines and omentum, which are also frequent sites of metastasis in human ovarian cancer patients (Naora and Montell 2005). Although it is possible that some of the peritoneal tumours result from leakage of adenovirus during surgical manipulation of the mice, previous studies have shown that the adenovirus is confined to the intrabursal space 7 days after intrabursal injection (Clark-Knowles, Garson et al. 2007). Moreover, at early time points (prior to the formation of nascent ovarian tumours), TAg expressing cells were found only in the OSE and bursal membrane. We did not see expression of TAg within the growing follicles or in the corpus luteum.

Epidemiological studies have shown that women are at an increased risk of developing ovarian cancer while using HRT (Lacey, Mink et al. 2002; Beral, Bull et al. 2007), after long-term use of HRT (greater than 5 years) (Lacey, Mink et al. 2002; Lacey, Brinton et al. 2006; Beral, Bull et al. 2007), and/or while using estrogen-only regimens (Lacey, Mink et al. 2002; Rossing, Cushing-Haugen et al. 2007). Although it has been convincingly shown that exogenous E_2 (such as HRT use) can increase the risk of developing ovarian cancer, it remains unclear how the hormone alters the etiology of the disease. A key finding of this paper is that the administration of E_2 in the tgCAG-LS-TAg model of ovarian cancer did not decrease survival by accelerating tumour growth, since E_2 treatment did not alter the proliferation rate of the malignant cells. Rather the shorter survival was the result of a decrease in the latency of the disease, reducing the period of time from induction of an oncogene until the onset of development of a micro tumour. These observations suggest that E_2 acts directly on the OSE cells, increasing their susceptibility to transformation. The mechanism for this action is unknown, but E_2 may accelerate the "second-hit" in OSE cells that are already primed to undergo transformation, likely by causing genomic instability due to rapid proliferation (Bai, Oliveros-Saunders et al. 2000; Syed, Ulinski et al. 2002; Stewart, S. L., Querec et al. 2004a; Cheung, Au et al. 2006) or oxidative stress (Symonds, Merchenthaler et al. 2008), and/or by inducing pathways of differentiation resulting in preneoplastic lesions (Bai, Oliveros-Saunders et al. 2000; Murdoch, Van Kirk et al. 2008).

In animals and in humans there is an increased incidence of putative preneoplastic lesions on the ovary following exposure to exogenous estrogens. In rats (Perniconi, Simoes Mde et al. 2008) and rabbits (Bai, Oliveros-Saunders et al. 2000) treatment with

estrogens has been shown to either cause a thickening of the OSE (Perniconi, Simoes Mde et al. 2008) or the formation of papillary structures on the OSE (Bai, Oliveros-Saunders et al. 2000). Likewise, ovarian surface papillomatosis was found to be more prevalent in women who had used HRT (Tok, Ertunc et al. 2006). Similarly, we noted differences in OSE morphology in the non-tumourigenic tgCAG-LS-TAg mice (AdGFP) treated with E_2, even without expression of the SV40 TAg oncogene. The presence of putative preneoplastic lesions, specifically areas of columnar cells or hyperplasia, in the OSE of E_2 treated mice confirms that exogenous E_2 acts not only on ovarian cancer cells, but also on the OSE cells. Ovarian tumours obtained from tgCAG-LS-TAg mice treated with E_2 histologically displayed many surface papillae which were rarely seen in the tumours of control mice given placebo pellets, suggesting that E_2 does not only affect epithelial morphology in normal ovaries, but may also have similar and long-lasting effects on the histology of ovarian tumours. In women, current users of HRT are at an increased risk of developing the most prevalent histological subtype of ovarian cancer, serous adenocarcinomas, as compared to the less common histologic subtypes (mucinous, endometrioid and clear cell) (Beral, Bull et al. 2007).

Although E_2 is able to alter the morphology of the OSE cells, the mechanism for this effect is unclear. The elevated levels of circulating E_2 may have indirect systemic effects, for example, altering the secretion of hypothalamic and pituitary hormones. Conceivably, E_2 could be acting directly on the OSE cells which express ERα and ERβ (Lau, K. M., Mok et al. 1999; Lindgren, Cajander et al. 2004), altering pathways of differentiation or enhancing susceptibility to transformation. Surprisingly, E_2 did not affect the *in vivo* proliferation of mouse OSE cells.

Treatment of the tumourigenic tgCAG-LS-TAg mice with P_4 resulted in a modest, albeit not statistically-significant increase in survival. *In vitro* experiments have shown that exogenous P_4 only inhibits proliferation of OSE and ovarian cancer cells at high concentrations (Syed, Ulinski et al. 2001). Increasing the dose or prolonging the duration of P_4 treatment in this mouse model may result in a greater increase in survival time. Moreover, Rodriguez *et al*. (Rodriguez, Walmer et al. 1998) showed that macaques given the progestin-only component of oral contraceptives had an increased incidence of apoptosis in the ovarian surface epithelium when compared to controls. Perhaps pre-treating the tgCAG-LS-TAg mice with P_4 prior to the induction of SV40 TAg would increase apoptosis in the OSE and prolong the latency of tumour onset in this mouse model.

In this study, we have generated a new mouse model of ovarian cancer that reliably develops poorly-differentiated ovarian tumours with a latency of approximately 90 days and a median overall survival of 113 days. Using this model, we have demonstrated that E_2 is able to accelerate the initiation of autochthonous ovarian cancer, resulting in a decreased survival time, whereas P_4 treatment did not affect survival. The ability of E_2 to elicit differences not only in survival time, but also in the histomorphology of the disease suggests that the hormone possesses the ability to stimulate distinct pathways of differentiation in the OSE. These results highlight the importance of E_2 on the onset and progression of ovarian cancer, and provide opportunities to examine the molecular mechanisms by which steroid hormones influence the etiology of the disease. More importantly, further studies using this model will lead

to a better understanding of the consequences of prolonged exposure to exogenous steroid hormones, such as those found in oral contraceptives and HRT.

Supplemental Data

Materials and Methods

RIAs

Blood samples were collected from the mice via either the lateral saphenous vein or cardiac puncture into heparinized vials (Sarstedt, Germany). The serum was separated via centrifugation (6000 rpm, for 10 minutes at 4°C) and stored at -20°C until use. Serum levels of E_2 and P_4 were determined by Coat-A-Count radioimmunoassay following the manufacturer's instructions (RIA, Diagnostic Products Inc.). Circulating levels of E_2 and P_4 at different time points were compared using a one-way ANOVA and Tukey's post-test.

Expression of SV40 TAg in the tgCAG-LS-TAg mouse OSE cells

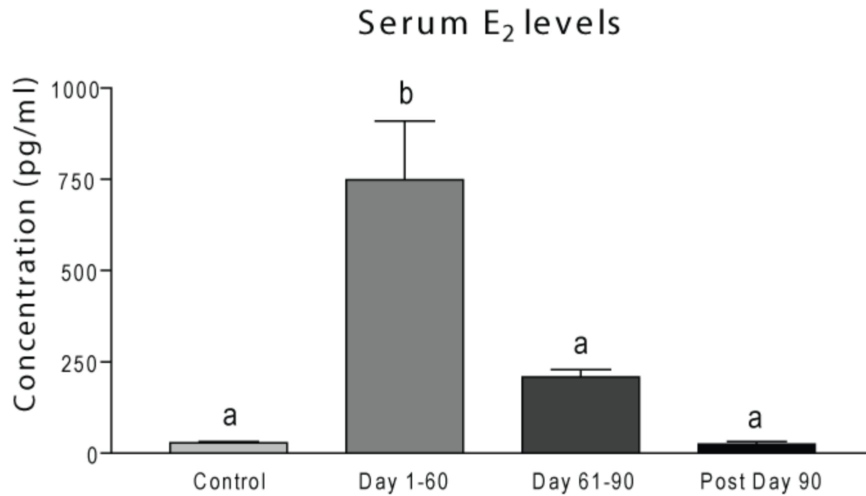
OSE cells were collected from tgCAG-LS-TAg mice and grown in culture following an established protocol (Clark-Knowles, Garson et al. 2007). Cells were pre-treated with either 500 nM E_2 or vehicle control (ethanol) for 10 days and then continuously treated for the duration of the experiment. The OSE cells were infected with AdCre (to induce recombination of the transgene and expression of SV40 TAg) or AdGFP (as a control) according to methods previously described (Flesken-Nikitin, Choi et al. 2003). Protein was collected and detected by Western blot using the SV40 TAg antibody (1:1000, Santa Cruz), according to previously described methods (Clark-Knowles, Garson et al. 2007).

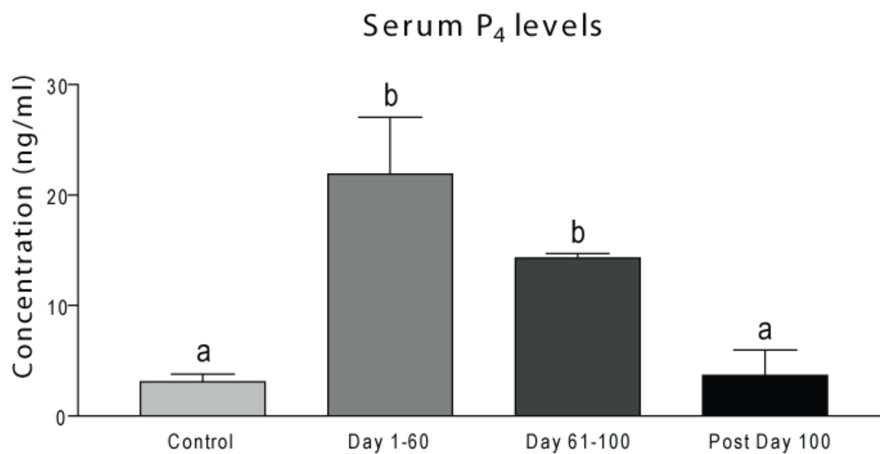
Cyclin D1 Primer Sequences

The following primer sequences were used for Cyclin D1 quantitative RT-PCR:

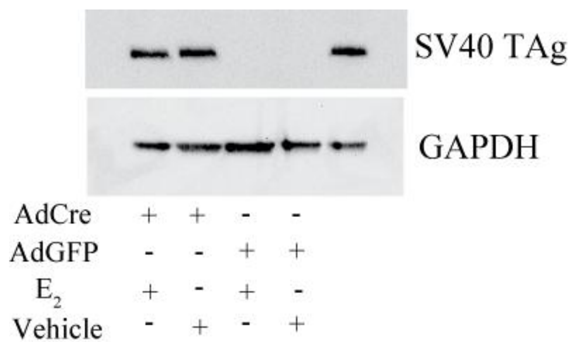
FWD - GGCTGCGATGCAAGGCCTGA

REV – CGACGTTCTGCTGGGCCTGG

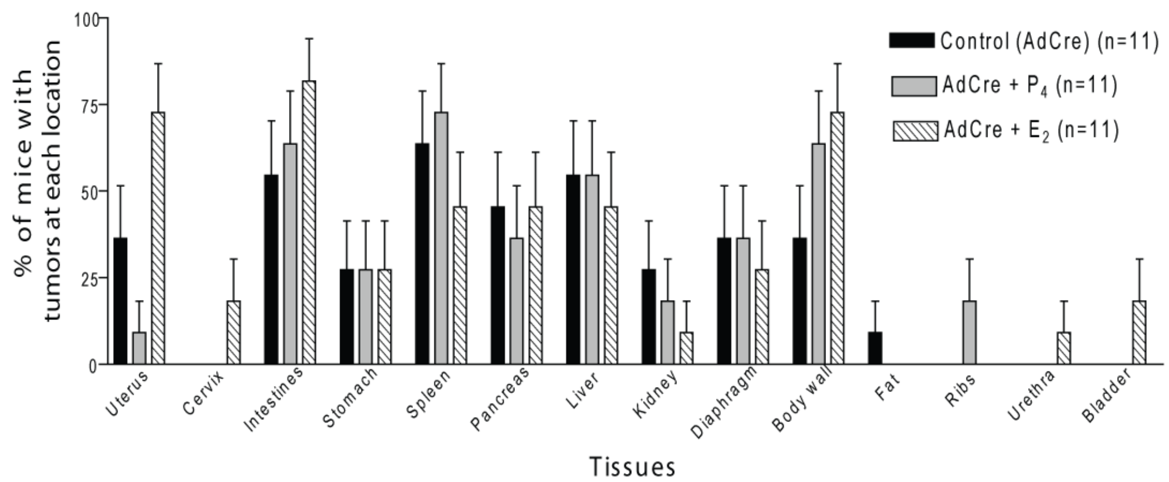




Supplemental Figure 1. The circulating steroid hormone levels in mice treated with either E_2, P_4, or controls as measured by RIA. The average level of serum E_2 was significantly increased following hormone implantation from 28.43 pg/ml (n=7) in controls to 748.38 pg/ml (n=6) between days 1-60 of pellet exposure. The average serum concentration of P_4 in the mice treated with P_4 pellets was elevated from 3.1 ng/ml (n=9) in controls to 21.9 ng/ml between days 1-60 of treatment (n=7) ($P<0.05$ a-b). Control refers to mice not treated with exogenous hormones and includes samples taken either before pellet insertion or from mice treated with a placebo pellet. Error bars indicate SEM.



Supplemental Figure 2. The expression of SV40 TAg in tgCAG-LS-TAg OSE cells. OSE cells were treated with either E_2 or vehicle control and protein was collected following an *in vitro* infection with AdCre or AdGFP. Western blotting was used to determine the expression level of SV40 TAg in the OSE cells. The last lane contains protein lysate from a cell line known to express the SV40 TAg protein, which was used as a positive control.



Supplemental Figure 3. The sites of tumour dissemination in the tgCAG-LS-TAg mice. Mice from each of the 3 treatment groups (AdCre + Placebo, AdCre + E_2, AdCre + P_4) had a substantial amount of tumour dissemination to several tissues within the peritoneal cavity. Error bars indicate SEM.

Supplemental Table 1. Immunophenotype of tgCAG-LS-TAg tumours treated with placebo, P_4 or E_2.

	pan-CK	CK19	Calretinin	S100α	CD45R	Inhibin-α	PLAP	Synapto
AdCre (control)								
1139RTumour	-	-	+	+	-	-	-	-
1140 LTumour	- (f)	-	-	+	-	+	-	-
1495 RTumour	+	-	-	+	-	+	-	-
3526 LTumour	-	-	-	+	-	+	-	-
AdCre + P_4								
1183 LTumour	-	-	-	+	-	+ (f)	+ (f)	-
1969 RTumour	+ (f)	-	-	+	-	-	-	-
3518 RTumour	-	+ (f)	-	+	-	+	-	-
3528 LTumour	-	-	-	+	-	+	-	-
AdCre + E_2								
1180 RTumour	+	N/A	-	+	-	+	-	-
1967 LTumour	+	-	-	+	-	-	-	-
1968 RTumour	+ (f)	N/A	-	+	-	+ (f)	-	-
1968 LTumour	+	N/A	-	+	N/A	-	-	N/A
1973 RTumour	-	+ (f)	+	+	-	+	-	-

Abbreviations: LTumour, left ovarian tumour; RTumour, right ovarian tumour; pan-CK, pan-cytokeratin; CK19, cytokeratin 19; PLAP, placental alkaline phosphatase; Synapto, synaptophysin; (f), focal staining; N/A, not available

Chapter 3: 17β-estradiol accelerates ovarian tumour progression *in vivo* through the upregulation of Greb1

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Abstract

Exogenous 17β-estradiol (E2) accelerates the progression of ovarian cancer in the tgCAG-LS-TAg mouse model of the disease. We have previously shown that this effect may be mediated in part by E2's ability to cause putative preneoplastic lesions in the ovarian surface epithelium resulting in an earlier onset of ovarian cancer. We hypothesized that E2 also has direct effects on ovarian cancer cells and this study was designed to determine the molecular mechanisms through which E2 accelerates ovarian tumour progression. Mouse ovarian cancer ascites cell lines (MAS) were derived from the tgCAG-LS-TAg mouse model. Treatment of these cells with 500nM E2 *in vitro* did not affect their proliferation or colony formation. Following engraftment of two MAS cell lines, MASC1 and MASE2, into SCID mice, exogenous E2 significantly decreased the survival time and increased the tumour burden. Microarray analysis performed on MASE2-derived tumours treated with and without E2 indicated that E2 treatment causes the upregulation of 197 genes and downregulation of 55 genes, mainly involved in cell differentiation, proliferation and migration. The expression of Gene regulated by estrogen in breast cancer 1 (*Greb1*) was upregulated in mouse tumours treated with E2 and was overexpressed in human ovarian tumours relative to human ovarian surface epithelium, suggesting a role for Greb1 in human ovarian tumour progression. These results emphasize the importance of E2 on ovarian tumour progression and reveal novel gene targets for therapeutic intervention.

Introduction

The 5-year survival rate for women diagnosed with epithelial ovarian cancer (EOC) is only 46% making it the most lethal of the gynaecologic malignancies (Jemal, Siegel et al. 2010). The gravity of the disease is mainly attributed to advanced stage at diagnosis, with 79% of ovarian cancers detected after disease metastasis (Jemal, Siegel et al. 2010), which can include the spread of cancer cells throughout the peritoneal cavity and the accumulation of abdominal ascites (Naora and Montell 2005). One of the risk factors for EOC is the use of exogenous steroid hormones after menopause, known as hormone replacement therapy (HRT). Several epidemiologic studies have indicated that the increased risk of developing EOC due to HRT use is mainly associated with estrogen-only HRT (Lacey, Mink et al. 2002; Glud, Kjaer et al. 2004) or current use of HRT (Beral, Bull et al. 2007; Rossing, Cushing-Haugen et al. 2007; Morch, Lokkegaard et al. 2009).

In addition to the epidemiologic evidence, several basic research studies have indicated that exogenous 17β-estradiol (E2) may increase the risk of developing EOC by acting directly on the ovarian surface epithelium (OSE), which is believed to be the tissue of origin for most EOCs. OSE cells treated with E2 may be more susceptible to transformation, due to E2's ability to increase proliferation (Bai, Oliveros-Saunders et al. 2000; Wright, J. W., Toth-Fejel et al. 2002), produce oxidative DNA damage (Symonds, Merchenthaler et al. 2008), and cause preneoplastic lesions in the OSE (Bai, Oliveros-Saunders et al. 2000; Laviolette, Garson et al. 2010). E2 increases proliferation of rhesus macaque OSE cells *in vitro* at concentrations comparable to the level in follicles just prior to ovulation (Wright, J. W., Toth-Fejel et al. 2002). Treating rabbits with E2 *in vivo*

increases the proliferation of the OSE cells and causes papillary structures on the OSE, which are putative preneoplastic lesions for EOC (Bai, Oliveros-Saunders et al. 2000). Moreover, we have previously shown that treating mice with E2 results in an increase in putative preneoplastic lesions in the OSE, including areas of hyperplasia (Laviolette, Garson et al. 2010).

Although E2 can act on the normal OSE cells to increase the risk of developing EOC, it can also accelerate EOC progression once tumours are initiated. E2 is both a mitogen and a cell survival factor for EOC cells and may affect cell adhesion, invasion and the ovarian tumour microenvironment. E2 increased thymidine incorporation in tumourigenic human OSE cells, which was attributed to an inhibition of apoptosis, rather than an increase in proliferation, likely mediated through the E2-induced upregulation of Bcl-2 (Choi, Kang et al. 2001). E2 has also been shown to reduce paclitaxel-induced apoptosis in the Caov-3 ovarian cancer cell line through alterations in the Akt/JNK signalling pathway (Mabuchi, Ohmichi et al. 2004). We have shown that exogenous E2 accelerates ovarian cancer progression, resulting in a decrease in overall survival time, in a transgenic mouse model of ovarian cancer (Laviolette, Garson et al. 2010). Additionally, a recent study indicated that the estrous cycle affects tumour growth in ovarian cancer xenografts, with increased tumour burden occurring in mice during the proestrous phase when E2 levels are the highest (Armaiz-Pena, Mangala et al. 2009). Similarly, E2 administration to ovariectomized mice inoculated with ovarian cancer cells significantly increased the tumour burden (Armaiz-Pena, Mangala et al. 2009).

E2's ability to enhance EOC progression may be mediated through changes in the tumour microenvironment. E2 increases the expression of the extracellular matrix

protein fibulin-1 in ovarian cancer cells, indicating a potential role of the hormone in modeling the tumour microenvironment (Bardin, Moll et al. 2005). An E2 metabolite, 4-hydroxy estradiol, induces hypoxia-inducible factor 1α (HIF-1α) and vascular endothelial growth factor A (VEGF-A) expression in ovarian cancer cell lines, suggesting that E2 can stimulate angiogenesis (Gao, Nester et al. 2004). Moreover, adhesion of EOC cells to collagen is augmented by E2 treatment in *in vitro* assays (Armaiz-Pena, Mangala et al. 2009) and E2 enhances migration and induces epithelial-mesenchymal transition in EOC cells through an upregulation of the transcription factors Snail and Slug, implying that the hormone plays a role in tumour metastasis (Ding, Feng et al. 2006; Park, S. H., Cheung et al. 2008).

Although the biological consequences of E2 on EOC etiology are diverse, the molecular mechanisms underlying these effects are not well characterized. Breast cancer research has identified some E2-inducible gene targets involved in tumour progression, including Gene regulated by estrogen in breast cancer 1 (*Greb1)* (Ghosh, Thompson et al. 2000). *Greb1* was first identified as a hormone-responsive gene in the breast cancer cell line MCF7 (Ghosh, Thompson et al. 2000). Although the function of Greb1 is still unknown, recent studies have indicated that it is an early response gene that is a key regulator of E2-stimulated breast cancer cell growth (Rae, Johnson et al. 2005). Greb1 expression in EOC has not been investigated, despite EOC being well-established as a hormone responsive cancer.

Previously, we demonstrated that exogenous E2 accelerates ovarian tumour progression in a transgenic mouse model of ovarian cancer, tgCAG-LS-TAg mice, by inducing an earlier onset of tumourigenesis that resulted in a decreased overall survival

time (Laviolette, Garson et al. 2010). In this study, we aimed to determine the molecular mechanisms by which E2 is able to accelerate EOC progression. Using mouse ascites (MAS) cell lines derived from the hormone-responsive tgCAG-LS-TAg model of ovarian cancer, we tested the ability of E2 to increase cell growth *in vitro* and *in vivo*. Although E2 treatment had little effect on *in vitro* MAS cell proliferation, it significantly accelerated tumour progression of MAS cell grafts *in vivo*. We performed microarray analysis on tumours derived from MAS cells grafted into immunodeficient mice treated with and without E2 to identify genes involved in E2-driven tumour growth. *Greb1* emerged as an E2-regulated molecular target in our study and was found to be expressed in both mouse tumours and human ovarian cancers. These results highlight the importance of E2 in EOC progression and identify *Greb1* as a novel mediator of this process.

Materials and methods

Generation of mouse ascites cell lines

Mouse ovarian cancer ascites cells (MAS) were obtained from a previously described transgenic mouse model of ovarian cancer (Laviolette, Garson et al. 2010). Briefly, the tgCAG-LS-TAg mouse model utilizes the Cre/*loxP* system to inducibly express the oncogene SV40 large and small T-Antigens (SV40 TAg) in the mouse OSE cells following the intrabursal injection of adenovirus expressing AdCre (as previously described, (Clark-Knowles, Garson et al. 2007; Laviolette, Garson et al. 2010)). At the time of necropsy, mouse ascites cells from the tgCAG-LS-TAg mice were collected via

needle aspiration of the abdominal ascites. The ovarian cancer cells were grown in "mOSE media" which is alpha minimum essential medium (α-MEM; Hyclone, Logan, UT) containing 10% fetal bovine serum (FBS, Cansera, Etobicoke, ON, Canada), 5U/mL penicillin/5μg/mL streptomycin solution (Sigma-Aldrich, St. Louis, MO), 0.1μg/mL gentamicin (Gibco-BRL), 0.01 μg/mL epidermal growth factor (R&D Systems), and 1 μg/mL insulin–transferrin–sodium selenite solution (ITSS; Roche). The cells were grown at 37°C and 5% humidity and were repeatedly passaged. Mycoplasma testing was performed regularly. PCR analysis confirmed that the cell lines expressed the oncogene SV40 TAg, indicating that the cell lines were derived from tgCAG-LS-TAg mouse ovarian cancer cells. In total, 6 cell lines were derived from the tgCAG-LS-TAg mice. MASC1 and MASC2 were obtained from the ascites of tgCAG-LS-TAg mice treated with a placebo pellet (as previously described in (Laviolette, Garson et al. 2010)), MASE1 and MASE2 were collected from the ascites of mice treated with E2, and MASP1 and MASP2 were obtained from mice treated with progesterone (P4). All of these cell lines were cultured in the absence of steroid hormones, unless otherwise indicated.

Cell proliferation assay

All six MAS cell lines were seeded at 15,000 cells per well in 6-well cell culture dishes (Corning Inc., Corning, NY) and 24 hours after plating, were treated with either 500nM of E2 (Sigma-Aldrich, St. Louis, MO) or the vehicle control (an equal volume of 100% ethanol) in mOSE media. After 48 hours of E2 treatment, the cells were counted using a Coulter Counter (Beckman Coulter, Mississauga, ON, Canada). For the steroid

hormone treatments, charcoal purification was used to eliminate any residual steroid hormones present in the FBS. Charcoal (50 mg/ml) was added to the FBS and stirred overnight at 4°C. To remove the charcoal, the FBS was centrifuged at 1800 x *g* for 30 minutes. The supernatant was collected and centrifuged at 27,000 x *g* for 60 minutes. The FBS was filter sterilized using a 0.22 µm filter and stored at 4°C until use.

Soft-agar colony formation assay

MASC1 and MASE2 cells were used in this assay along with the human ovarian cancer cell line A2780cp (which served as a positive control) and mouse OSE cells (negative control). Two concentrations of UltraPure low melting point (LMP) soft agar (Invitrogen) were prepared and sterilized by autoclaving - a base layer of 2% LMP agarose (mixed with ddH_2O) and a top layer of 1% LMP agarose. The soft agar was heated in a microwave and was then kept at 37°C during use. Equal volumes of soft agar and 2x Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) media (phenol red free, Sigma-Aldrich, St. Louis, MO) containing 5% charcoal purified FBS, were mixed together and either E2 (250nM) or vehicle control (100% ethanol) was added to the mixture. Base soft agar (2%, 300µL) was pipetted into each well of a 24-well tissue culture dish. Cells were added to the top layer soft agar (1%) at 6 dilutions (2000, 1000, 500, 250, 125, or 62.5 cells/well). Each of the dilutions was plated in quadruplicate (in 700µL of soft agar). The colonies grew for two weeks and two dilutions (8 wells) per cell line were counted and expressed as a percentage of the input cells.

MAS cell grafts into immunodeficient SCID mice

MASC1, MASC2 or MASE2 cells (10^7 cells/animal) were injected intraperitoneally (IP) into normal (5-6 weeks old) or ovariectomized (OVX, 8-9 weeks old) female Fox Chase SCID mice (CB17-*Prkdc*^{scid}/NCrCrl, Charles Rivers Laboratories, Montreal, QC). Five days after the IP injection of MASC2 and MASE2 cells, a 60-day slow release hormone pellet (Innovative Research of America, Sarasota, FL) was surgically implanted (subcutaneously in the neck region). Mice were treated with either E2 (0.25 mg/pellet) or a placebo pellet (N=7-9 mice per group). The OVX mice grafted with either MASC1 or MASE2 cells were treated with either an E2 (0.25 mg/pellet) or a placebo pellet concomitantly with the IP injection of cells (N=5-7 mice per group). Mice were housed in sterile conditions on a 12L:12D cycle, with free access to food and water and were euthanized when they reached a loss-of-wellness endpoint. The survival time is the amount of time from the day the cells were grafted (injected IP) until the day when the mice reached a loss-of-wellness endpoint including: weight loss > 15 % of body weight, body weight increase > 5 g with respect to age-matched controls, palpable tumours (2 cm in size), presence of abdominal distension that impairs mobility or affects wellness, respiratory distress, dehydration, and/or anorexia. All animals were euthanized via CO_2 asphyxiation. All animal experiments described in this study were performed according to the *Guide to the Care and Use of Experimental Animals* established by the Canadian Council on Animal Care.

Preparation and analysis of tissues

Tissues were collected from mice at necropsy, and the volume of ascites and wet weight of the total tumour burden were recorded. Tissues were either flash frozen (for RNA and protein collection), or fixed in 10% neutral-buffered formalin overnight, transferred to 70% ethanol, and paraffin-embedded. For histological analyses, 3-5 μm sections were cut and stained with hemotoxylin and eosin (H&E). Tissue sections were scanned and imaged using the Aperio ScanScope and the Aperio ImageScope program (Aperio Technologies, Inc., Vista, CA, USA). Tumour proliferation was determined by performing immunohistochemistry on the tumours with the proliferation marker Ki67 (1:25, DAKO) as previously described (Laviolette, Garson et al. 2010). Positive pixel counting was performed to detect Ki67 positive nuclei using the Aperio ImageScope program (Aperio Technologies, Inc., Vista, CA, USA). Four random fields of view (200x magnification) were counted and averaged to obtain a mean for each tumour. At least four tumours per group (MASC1 + placebo, MASC1 + E2, MASE2 + placebo and MASE2 + E2) were stained and counted.

Microarray analysis

RNA was extracted from MASE2 tumours grown in SCID mice treated with or without E2 using the RNeasy kit (Qiagen) according to the manufacturer's instructions. Three biological replicates were analyzed for both conditions (addition of E2 or placebo) on Affymetrix microarray platform Mouse Gene 1.0 ST Array which measures the expression of approximately 28,850 genes. Signal intensities were normalized and summarized with variance stabilization normalization (Huber, von Heydebreck et al.

2002) and RMA (Irizarry, Bolstad et al. 2003), respectively, and probe sets differentially expressed between both conditions (E2 and placebo) were detected with limma (Smyth 2004). Gene functions were analyzed with Gene Ontology (GO) terms annotations from the MGI and GOA for mouse and human, respectively, and orthologs between both species mapped by NCBI's Homologene (Sayers, Barrett et al. 2010). Pathway annotations were obtained from the Kyoto Encyclopedia of Genes and Genomes (Kanehisa, Goto et al. 2010).

RNA extraction and Quantitative RT-PCR

Quantitative RT-PCR (Q-PCR) was used to determine the amount of ERα, ERβ and Greb1 mRNA in the MAS cell lines and human ovarian cancer cell lines treated with E2. MASC1, MASE2, A2780cp, HEY, and ES-2 cells were grown in DMEM/F12 (phenol red free) with 5% serum and either 500nM E2 or the vehicle control (100% ethanol) for 48 hours. The cells were collected via trypsinization and the cell pellets were flash frozen and stored at -80^{0}C for future RNA extraction. To validate the microarray target genes *Greb1*, *Cyp11a1*, *Stc2*, and *PgR,* RNA was extracted from MASE2 tumours grown in OVX SCID mice treated with and without E2 (n=4-5 tumours/group). RNA was extracted from the cultured cells and the tumours using the RNeasy Kit (Qiagen) and cDNA was prepared using the Superscript III First-Strand Synthesis System for RT-PCR kit (Invitrogen) according to the manufacturers' instructions. cDNA (100 ng per cell line or tumour) was used to determine expression of *ERα, ERβ, Greb1, Cyp11a1, Stc2*, and *PgR* relative to mouse whole ovary cDNA, with 18S used as an endogenous control. For the human cancer cell lines, *GAPDH* was used as the endogenous control and expression

of *Greb1* was expressed relative to MCF7 cDNA (positive control). Q-PCR reactions were set up in 96-well plates and run on the Applied Biosystems 7500 Fast RT-PCR system using SYBR green fast reaction settings (ABI, Carlsbad, California). Data were analyzed using the ABI 7500 software (version 2.0.1). The primers were used at a concentration of 10 µM and the sequences were as follows: mERα FWD TCTGCAGCAGCAGCATCGCC; mERα REV GGCATGAAGGCGGTGGGCAT (product size 166 bp); mERβ FWD CGTTCTGGACAGGGATGAGGGGA; mERβ REV GGCTTGCGGTAGCCAAGGGG (product size 173 bp); mGreb1 FWD GCAACACGGTGCCTCCACCA; mGreb1 REV GAGGCGCCTGCTGGTACTGC (product size 182 bp); mCyp11a1 FWD CATGCGAGGGTCCCAACCCG; mCyp11a1 REV CACCTTCCAGCAGGGGCACG (product size 186 bp); mStc2 FWD GAGCGGCTGGGCCAGTTTGT; mStc2 REV CCACGTCCCCGGCATTGACC (product size 196 bp); mPgR FWD CGCAGCACCCGCCATCTACC; mPgR REV ACTGTGGGCTCTGGCTGGCT (product size 152 bp); r18S FWD CGCGGTTCTATTTTGTTGGT; r18S REV AGTCGGCATCGTTTATGGTC (product size 219bp); hGreb1 FWD TAGCGACCCCTGGCCAGACC; hGreb1 REV GCCGTCTGACGCCGCACATA (product size 174 bp); hGAPDH FWD GCCGTCTGACGCCGCACATA; hGAPDH REV AGGCCATGCCAGTGAGCTTCC (product size 172 bp) and hERα FWD CCTGATGGCCAAGGCAGGCC; hERα REV CGGTGGGCGTCCAGCATCTC (product size 180 bp).

Human ovarian cancer samples

Greb1 mRNA levels were measured in human OSE and ovarian cancer tissue samples. RNA was extracted from three primary human OSE cell cultures (C19, C14 and C26 cells) at early passage. Four samples of snap frozen primary tumour tissue were obtained from the Ottawa Ovarian Cancer Tissue bank under a protocol approved by The Ottawa Hospital Research Ethics Board. These samples were obtained with informed consent from four patients with different histological subtypes of ovarian cancer: serous, endometrioid, mucinous and clear cell. RNA was extracted from each sample and cDNA was prepared as described above. The level of Greb1 mRNA expression in human tissue samples was measured by Q-PCR as described above.

Statistical analyses

All data were plotted using GraphPad Prism software (version 3.02: GraphPad Software, San Diego, CA, USA). MAS cell proliferation *in vitro*, *ERα, ERβ*, and *Greb1* expression *in vitro*, and Ki67 proliferation in tumours were compared using a One-Way ANOVA with a Tukey's post-hoc test. Colony formation was compared using a *t* test. Kaplan-Meier survival curves were compared using a Logrank test. Mouse tumour weights and ascites volumes were compared using *t* tests. Q-PCR validation of *Greb1, Cyp11a1, Stc2,* and *PgR* was compared using a *t* test. Significance was inferred at $P < 0.05$.

Results

E2 treatment does not affect the growth of mouse ascites cells *in vitro*

Mouse ascites cell lines (MAS) were derived from the abdominal ascites of tgCAG-LS-TAg mice with ovarian tumours. The ascites fluid was collected via needle aspiration and the ovarian cancer cells were grown in culture to establish cell lines. Each of the MAS cell lines was derived from an individual mouse that was treated *in vivo* with either placebo (MASC1 and MASC2 cell lines), P4 (MASP1and MASP2 cell lines) or E2 (MASE1 and MASE2 cell lines). In order to determine if MAS cell proliferation could be stimulated by E2, the cells were treated with 500 nM E2 or equal volumes of vehicle control for 48 hours. E2 did not affect the proliferation of any of the cell lines compared to the vehicle controls (Figure 1A). All of the cell lines grew at comparable rates, except for MASP1 cells treated with E2 which proliferated more in 48 hours than the MASE2 cells treated with E2 (Figure 1A). Moreover, E2 did not affect the growth of any of the MAS cell lines after 24, 72, or 96 hours compared to controls (data not shown).

Soft-agar colony formation assays were used to determine if E2 enhanced anchorage-independent growth of MAS cells. The MASC1 and MASE2 cells formed colonies with an efficiency of approximately 1% after 2 weeks (Figure 1B, inset). E2 treatment (250 nM) did not affect the ability of the MAS cells to form colonies. The human ovarian cancer cell line, A2780cp, was used as a positive control for the assay and these cells produced colonies with 80-90% efficiency (Figure 1B). Mouse OSE cells were used as a negative control for the assay, with only about 0.1% of the cells forming colonies (Figure 1B, inset). Neither A2780cp nor mouse OSE cells produced more or

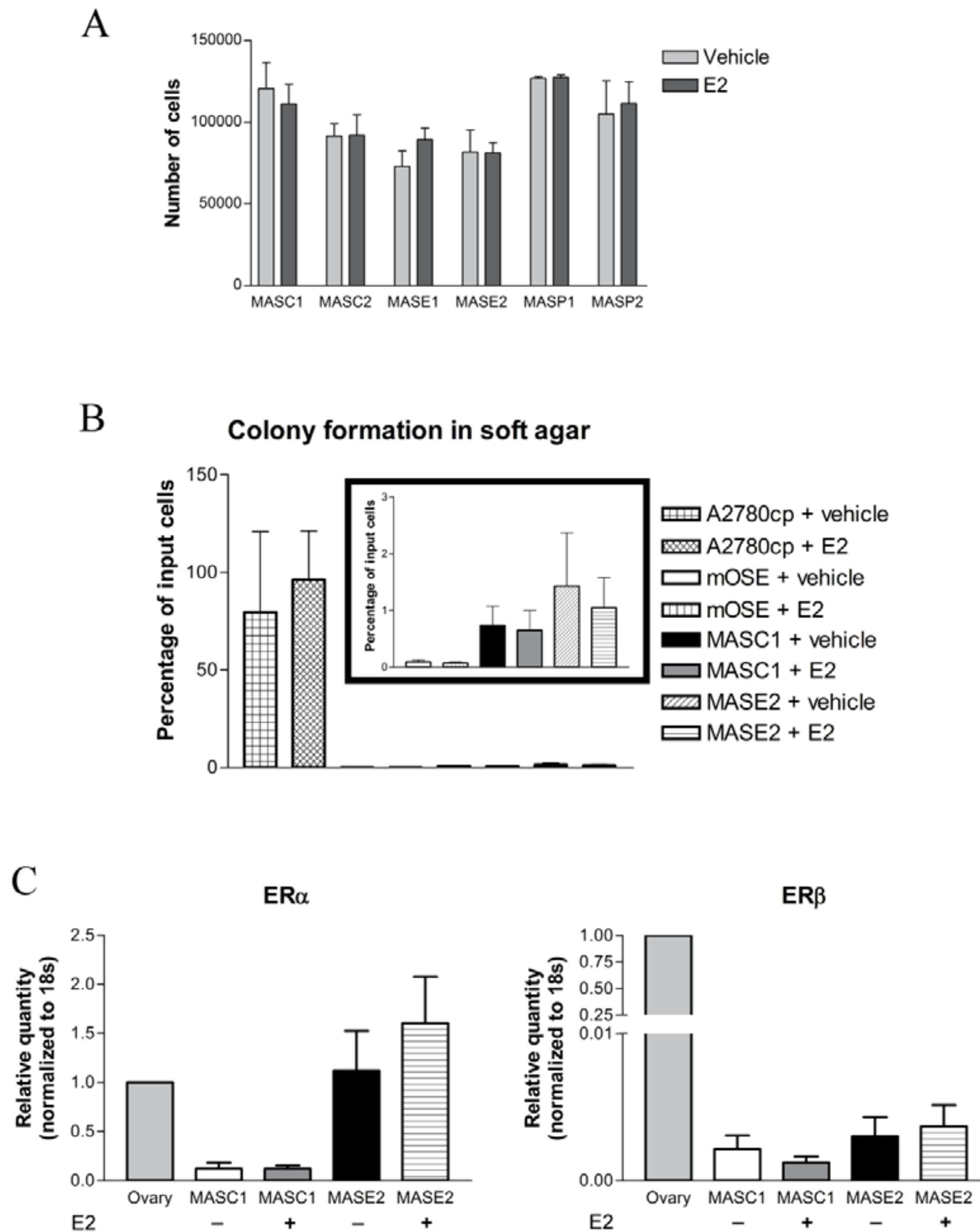


Figure 1. E2 treatment did not affect *in vitro* proliferation of mouse ovarian cancer cells. A) All of the mouse ascites cell lines proliferated at the same rate and treating them with 500 nM of E2 did not alter the rate of proliferation over 48 hrs (compared to those treated with vehicle). MASP1 cells treated with E2 proliferated significantly faster than MASE2 cells treated with E2 (n=3; $P<0.05$, One-Way ANOVA). B) E2 (250 nM) did not affect the ability of MASC1 and MASE2 cells to form colonies in soft agar (n=3; inset is a close up of the mouse OSE, MASC1 and MASE2 data). C) MASE2 cells had higher ERα mRNA levels than MASC1 cells (n=3, $P<0.05$, One-Way ANOVA and Tukey post-hoc test). ERα mRNA was not induced by treatment with E2 (500nM). ERβ expression was very low in all of the cell lines and was not induced by E2 treatment.

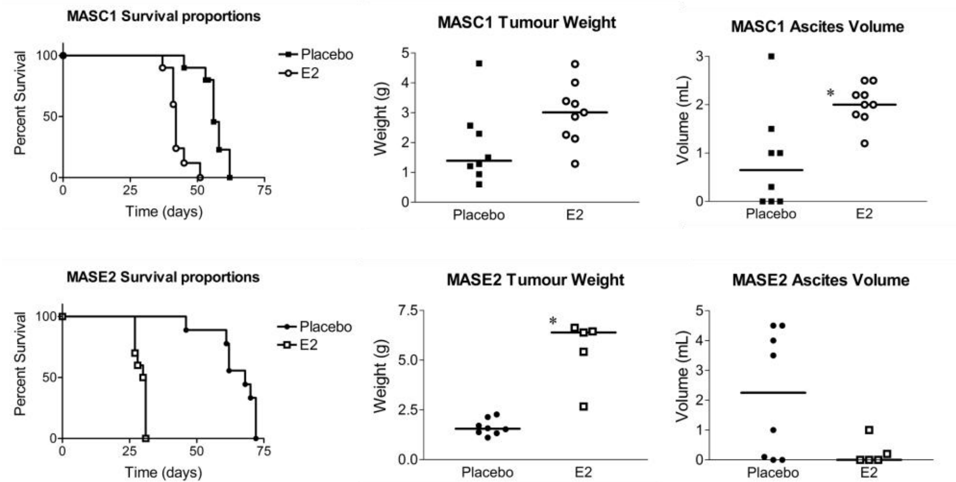
fewer colonies in response to E2 treatment (Figure 1B).

Because the MAS cells did not proliferate in response to E2 treatment *in vitro*, Q-PCR was used to determine whether the hormone receptors ERα and ERβ were expressed in the cultured cells. *ERα* was expressed in the MASE2 cell line at a level comparable to the positive control, whole mouse ovary, and the level of expression was not enhanced by E2 (500 nM for 48 hrs *in vitro*; Figure 1C). Similarly, *ERα* expression was not increased by E2 in the MASC1 cell line. The MASE2 cells treated with E2 had the highest level of *ERα* mRNA which was significantly higher than the levels found in the MASC1 cells alone or treated with E2 (Figure 1C). *ERβ* mRNA expression was very low in both the MASC1 and MASE2 cell lines *in vitro* compared to the mouse ovary and was not increased by E2 treatment (Figure 1C).

E2 treatment accelerates tumour progression *in vivo*

In order to determine the effects of E2 treatment on tumour growth *in vivo*, two of the MAS cell lines, MASC1 and MASE2, were grafted into immunodeficient SCID mice. The MAS cells express the oncogene SV40 TAg, which is highly immunogenic, therefore the cells were grafted into immunodeficient SCID mice instead of syngeneic hosts. 10^7 MASC1 or MASE2 cells were injected intraperitoneally into female SCID mice (5-6 weeks old). Five days after the cells were grafted into the mice, a hormone pellet was implanted subcutaneously to deliver E2 (0.25mg/pellet) for 60 days. A placebo pellet was inserted subcutaneously into the control mice. The delay of 5 days before treating the mice with hormone was designed to eliminate any effects of E2 on implantation of

A



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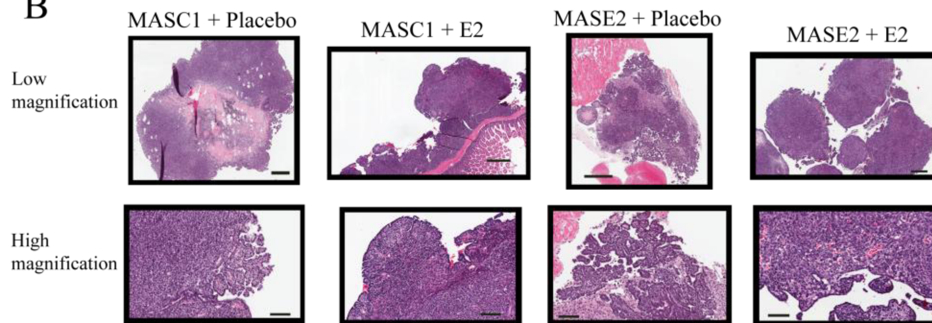

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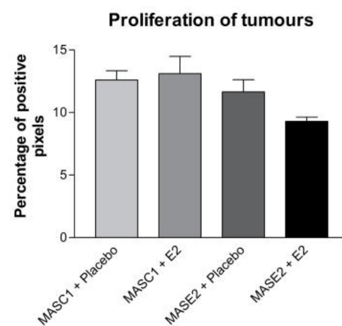


Figure 2. Exogenous E2 treatment decreased the survival time of immunodeficient mice grafted with mouse ascites cell lines MASC1 and MASE2. A) MASC1 or MASE2 cells were injected IP into SCID mice. Mice treated with E2 had a significantly shorter survival time compared to those treated with a placebo (*, MASC1 survival curve, P<0.0001, N=9 mice/group; MASE2 survival curve P=0.0004, Logrank test, N=7-8 mice/group). Mice grafted with MASC1 cells and treated with E2 had significantly elevated volumes of ascites compared to placebo controls (*, P=0.007, *t* test, N=8-9). The tumour burden of mice grafted with the MASE2 cells and treated with E2 was significantly more than the placebo controls (*, P<0.0001 *t* test, N=5-8). B) The tumours from grafted MASC1 and MASE2 cell lines were poorly differentiated and highly mitotic. Low magnification scale bars, 500 µm; high magnification scale bars, 100 µm. C) E2 treatment did not affect the proliferation (Ki67 immunoreactivity) of ovarian cancer cells *in vivo* (One-way ANOVA, N=4-5 tumours/group).

the cancer cells. For both cell lines, MASC1 and MASE2, E2 treatment significantly accelerated the progression of tumourigenesis (Figure 2A). SCID mice grafted with MASC1 cells and treated with E2 had a significantly shorter median survival time compared to the placebo controls (42 vs. 56 days; Figure 2A; $P<0.0001$, Logrank test, N=9 mice/group). There was a trend toward an increase in tumour burden ($P=0.07$, *t* test) and a significant increase in ascites volume in mice treated with E2 compared to controls ($P=0.007$, *t* test; Figure 2A). The mean tumour weight at disease endpoint in mice receiving a placebo pellet was 1.88 ± 0.4590 g compared to 2.99 ± 0.3367 g in mice treated with E2 (Figure 2A). The majority (63%) of the control mice injected with MASC1 cells developed ascites with a mean volume of 0.85 ± 0.3674 mL, while 100% of the E2 treated mice had peritoneal ascites with a mean volume of 2.02 ± 0.1354 mL (Figure 2A).

Similarly, SCID mice grafted with MASE2 cells and treated with E2 had a significantly shorter median survival time compared to the placebo controls (30.5 vs. 68 days; Figure 2A; $P=0.0004$, Logrank test, N=7-8 mice/group). E2 treatment significantly increased the tumour weight in mice grafted with MASE2 cells and there was a trend toward a decrease in the incidence and volume of ascites in mice treated with E2 (Figure 2A). The mean tumour weight in placebo-treated mice was 1.63 ± 0.1409 g, and was increased to 5.51 ± 0.7412 g in E2-treated mice ($P<0.0001$, *t* test). The mean volume of ascites was 2.2 ± 0.7445 mL (75% of mice) in the placebo-treated mice and only 0.24 ± 0.1939 mL (40% of the mice) in mice given E2 (Figure 2A). Similarly, OVX immunodeficient SCID mice grafted with the MASC2 or MASE2 cells had a decreased survival time when treated with E2 (Supplemental Figure 1). In the absence of

endogenous ovarian hormones, exogenous E2 treatment was able to accelerate tumourigenesis (Supplemental Figure 1).

Histological examination of the tumours from the SCID mice revealed aggressive, poorly differentiated carcinomas with a high mitotic grade (Figure 2B). Areas of necrosis were apparent in the tumours (Figure 2B, low magnification - MASC1 + Placebo, MASE2 + Placebo) and papillary structures, reminiscent of the serous histological subtype of EOC, were seen at the edge of the tumours from all of the groups (Figure 2B). Immunohistochemistry for the proliferation maker Ki67 was used to determine whether E2 affected the growth rate of MAS cancer cells *in vivo*. The tumours were collected from the mice at disease endpoint, when the carcinomas should be at the most aggressive stage. E2 did not affect the proliferation of the MAS cells *in vivo* (Figure 2C), as determined by the percentage of positive pixels (cells expressing Ki67) in tumours from at least 4 mice per group (SCID mice grafted with MASC1 or MASE2 cells and treated with E2 or placebo; One-Way ANOVA and Tukey's post-test; Figure 2C).

E2 increases expression of genes involved in cell differentiation and migration

The molecular mechanisms through which E2 accelerates ovarian cancer progression were investigated by performing gene expression profiling on tumours from mice treated with either placebo or E2 pellets. Microarray analysis was performed on mouse tumours derived from the MASE2 cell grafts, because this cell line exhibited the greatest *in vivo* growth response to E2 (Figure 2A). Tumours obtained from three mice treated with E2 were compared to the tumours from three placebo-treated mice. E2

Table 1. Genes differentially expressed in mouse ovarian tumours treated with E2 (compared to placebo).

Upregulated by E2

Gene		**Gene Ontology Pathway**	**logFC**	**Adjusted P value**
Cyp11a1	Cytochrome P450 (scc)	Steroid metabolic process	4.26	0.0006
Greb1	Gene regulated by estrogen in breast cancer protein	Unknown	3.41	0.0015
Fgf7	Fibroblast growth factor 7	Cell proliferation Cell division	2.65	0.0044
Pgr	Progesterone receptor	Reproduction Transcription Cell proliferation Cell differentiation	2.64	0.0446
Cdh2	N-cadherin	Cell differentiation Cell migration	2.18	0.0044
Ednra	Endothelin receptor type A	Cell differentiation Angiogenesis	2.11	0.0281
Kdr	Vascular endothelial growth factor receptor 2	Cell proliferation Cell migration Cell differentiation Angiogenesis	2.07	0.0170
Stc2	Stanniocalcin 2	Biological process	1.90	0.0201
Bmp6	Bone morphogenetic protein 6	Cell differentiation Inflammatory response	1.31	0.0375

Downregulated by E2

Gene		**Gene Ontology Pathway**	**logFC**	**Adjusted P value**
Sgpp1	Sphingosine-1-phosphate phosphatase 1	Apoptosis	-0.85	0.0281
Met	Met proto-oncogene	Cell differentiation Cell migration Cell proliferation	-1.18	0.0442
Ptgs1	Cyclooxygenase-1	Cell proliferation	-1.47	0.0315

Abbreviations: logFC - logarithmic fold change

treatment resulted in a statistically significant upregulation of 197 genes and a downregulation of 55 genes. Gene ontology analysis revealed that many of the upregulated genes belonged to pathways involved in cell differentiation, cell proliferation, cell migration, angiogenesis and the steroid metabolic process, such as *Greb1*, *PgR*, *Cyp11a1*, *Cdh2*, *Fgf7*, and *Bmp6* (Table 1). MASE2 tumours treated with or without E2 obtained from a separate study, the OVX SCID mouse study (Supplementary Figure 1), were used to validate a subset of the microarray targets, including *Greb1*, *Cyp11a1, PgR* and *Stc2,* by Q-PCR. *Greb1* and *Cyp11a1* were significantly elevated and there was a trend toward increased levels of *PgR* and *Stc2* in tumours treated with E2 compared to the placebo controls (Figure 3). The gene expression levels in the mouse tumours were expressed relative to the levels in whole mouse ovary, which was used as a positive control. Both *PgR* and *Stc2* were elevated in E2-treated tumours relative to normal ovary.

Greb1* is expressed in human and mouse ovarian cancers and can be induced in cells that express *ERα

Greb1 was expressed in tumours from each of the histological subtypes of EOC, including serous, endometrioid, clear cell and mucinous, but was almost undetectable in normal human OSE cells (Figure 4A). *Greb1* mRNA was also present at low levels in the human ovarian cancer cell lines HEY, ES-2 and A2780cp (Figure 4B). Each of these cell lines exhibited very low to undetectable levels of *ERα* mRNA and *in vitro* treatment of these cells with E2 did not increase expression of *ERα* or *Greb1* (Figure 4B). *Greb1*

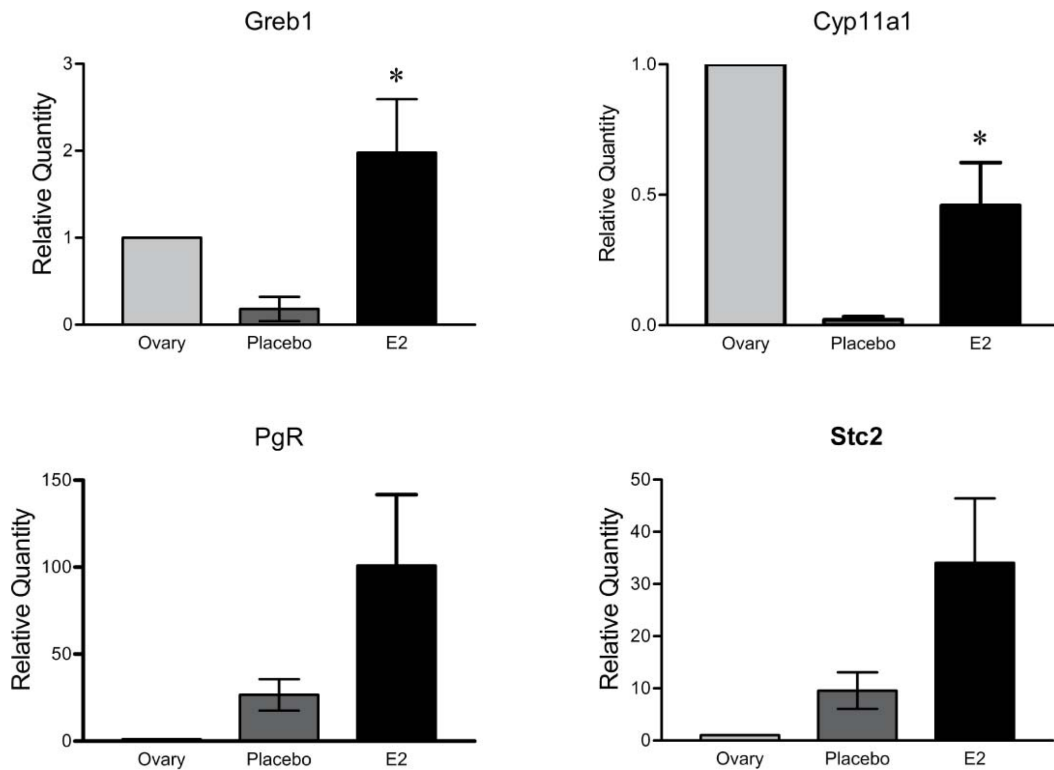


Figure 3. Q-PCR validation of microarray targets in MASE2-derived tumours treated with and without E2. *Greb1* (*, *P*=0.02, *t* test; n=4-5 tumours/group) and *Cyp11a1* (*, *P*=0.02, *t* test) gene expression was significantly increased in tumours from mice treated with E2 compared to controls. There was a trend toward increased mRNA expression of *Stc2* (*P*=0.07, *t* test) and *PgR* (*P*=0.09, *t* test) in E2 treated tumours compared to placebo controls.

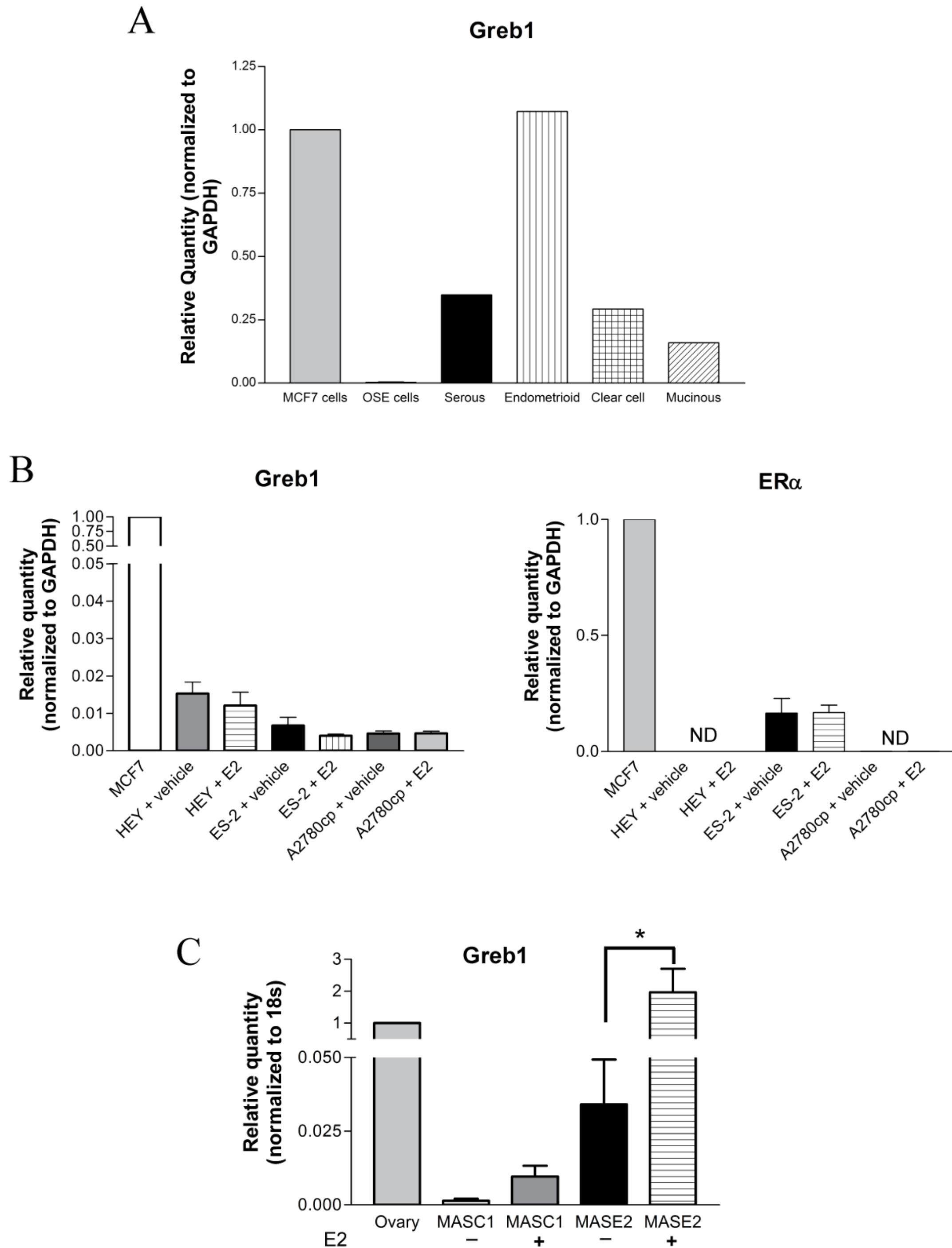


Figure 4. *Greb1* expression in human and mouse ovarian tumour tissue. A) *Greb1* was highly expressed in a sampling of all of the epithelial ovarian carcinoma subtypes, including serous, endometrioid, clear cell and mucinous, but was almost absent in human OSE cells. MCF7 breast cancer cells were used as a positive control for *Greb1* mRNA expression. B) *Greb1* was expressed in the human ovarian cancer cell lines HEY, ES2 and A2780cp, but was not induced by E2 treatment *in vitro*. *ERα* mRNA levels were very low in ES-2 cells and were non-detectable (ND) by Q-PCR in HEY and A2780cp cells. C) *Greb1* mRNA was present in MASC1 and MASE2 cells and was significantly increased in the MASE2 cells following treatment with 500 nM E2 for 48 hours *in vitro* (*, $P<0.05$).

was also detected in the mouse ascites cell lines MASC1 and MASE2 (Figure 4C). MASE2 cells treated with E2 (500 nM) for 48 hours exhibited a greater than 50-fold increase in *Greb1* expression compared to the vehicle controls ($P<0.05$, One-Way ANOVA, Figure 4C). MASC1 cells had very low levels of *Greb1*, which were not altered by E2 treatment (Figure 4C).

Discussion

The ability of E2 to stimulate ovarian tumour growth and the molecular mechanisms underlying this process are currently not well characterized. In this study, we have shown that E2 has little effect on ovarian cancer cell growth *in vitro*, but has the ability to significantly increase the growth of tumours *in vivo*. Microarray analysis suggested that the rapid growth of ovarian tumours in mice treated with E2 may be due to alterations in angiogenesis, cell differentiation, proliferation and migration, signifying the importance of the tumour microenvironment in ovarian cancer progression. *Greb1*, in particular, emerged as a novel gene altered by E2 in ovarian cancer, but has previously been reported to enhance the growth of breast and prostate cancer cells (Rae, Johnson et al. 2005; Rae, Johnson et al. 2006). We have shown that *Greb1* is expressed in mouse and human ovarian tumours and is upregulated by E2 treatment both *in vitro* and *in vivo*. These results underline the importance of reproductive hormones in EOC progression and provide new molecular targets to be investigated as mediators of E2-driven ovarian tumourigenesis.

Previously we reported that E2 accelerated ovarian tumour progression in the transgenic tgCAG-LS-TAg mouse model of ovarian cancer (Laviolette, Garson et al.

2010). This effect was attributed, in part, to an earlier onset of ovarian tumours in mice treated with E2 which resulted in a reduction in the overall survival time. Moreover, our previous results demonstrated that exogenous E2 altered the morphology of mouse OSE cells and increased the areas of putative preneoplastic lesions in the OSE (columnar cells and hyperplasia), which may render them more susceptible to transformation (Laviolette, Garson et al. 2010). In that previous study, it was unclear whether E2 was also able to affect the growth of ovarian tumours once the tumours were initiated, since the progression of the disease in the tgCAG-LS-TAg mice was very rapid (Laviolette, Garson et al. 2010). In this study, we aimed to determine the effects of E2 on ovarian cancer cell growth and to elucidate the molecular mechanisms through which E2 modulates ovarian cancer progression.

Using cell lines established from mouse ovarian cancer ascites (MAS cells), we have shown that E2 treatment of MAS cells *in vitro* does not affect the proliferation of the cells or the growth of colonies in soft agar. E2's limited capacity to affect proliferation *in vitro* may be due to different responses of ovarian cancer cells in the primary tumours vs. those found in the ascites, from which the cell lines were derived. There is also variable expression of the hormone receptors ERα and ERβ in the cultured MAS cell lines compared to the high expression of ERα, as evidenced by immunohistochemical staining, in the original tgCAG-LS-TAg mouse ovarian tumours (Laviolette, Garson et al. 2010). Although *ERα* was detected in both the MASC1 and MASE2 cells *in vitro,* the expression was much higher in the MASE2 cell line, which had levels similar to the mouse ovary. Interestingly, the expression of *ERβ* in the MASC1 and MASE2 cells *in vitro* was almost undetectable, suggesting that ERα plays a more

prominent role than ERβ in E2-mediated ovarian cancer progression. In addition to E2's established role as a mitogen, previous studies have indicated that E2 aids in cell survival and the ability of OSE and ovarian cancer cells to evade apoptosis (Murdoch and Van Kirk 2002; Mabuchi, Ohmichi et al. 2004). Mechanistically, this effect may be due to E2's ability to upregulate Bcl-2, a key anti-apoptotic protein (Choi, Kang et al. 2001). Conceivably, the effects of E2 on cell survival may be more evident during *in vivo* tumour growth, where challenges to cell survival may be more common (as seen by the areas of tumour necrosis) than is usually found in normal cell culture conditions.

The effect of E2 on ovarian tumourigenesis may be mediated by direct actions on the tumour cells and/or indirect actions on the ovaries or other E2-responsive tissue. The E2-stimulated acceleration of tumour progression in the OVX mice suggests that the E2 actions are independent of ovarian factors. The ability of E2 to accelerate the progression of tumourigenesis *in vivo*, but not cell growth *in vitro*, suggests that E2's effects on ovarian cancer cells are not directly mitogenic and may alternatively be dependent on the tumour microenvironment. Indeed, microarray analysis of the ovarian tumours derived from the same cell line, but treated with and without E2, showed that E2 stimulated an enrichment of gene expression changes in pathways such as cell differentiation, migration, and angiogenesis. E2 was also able to increase the growth rate and size of ovarian tumours, with those mice treated with E2 developing the largest tumour burden. Surprisingly, Ki67 immunostaining of the tumours did not reveal any differences in cellular proliferation within the tumours. This may be attributed to proliferation being measured in tumours collected from mice at a loss-of-wellness endpoint when the

carcinomas have reached their most aggressive stage and when the cancer cell proliferation may be at the maximum rate possible.

A previous study designed to examine the transcriptional profiles of human OSE and EOC cells treated with E2 *in vitro* revealed gene expression changes in pathways such as signal transduction, protein modification, apoptosis, protein biosynthesis, transcription, cell cycle progression, differentiation, and cell adhesion (Syed, Zhang et al. 2005). The study focused on five E2-regulated genes, *RNPS1*, *ADD1*, *rap-2*, *SKIIP*, and *PLXNA3,* that were differentially expressed in cultured EOC cells compared to non-malignant OSE cells (Syed, Zhang et al. 2005). Although expression of these five genes was not altered in our study, there was consistency between the two gene array studies in some of the pathways impacted by E2 in ovarian cancer, such as cell differentiation, transcription, apoptosis and cell adhesion. Moreover, a microarray study examining genes regulated by E2 in ERα positive human ovarian cancer xenografts indicated that exogenous E2 increases expression of genes involved in cell motility, such as *palladin* and *fascin 1* (Spillman, Manning et al. 2010). These studies, including ours, would suggest that while similar pathways are affected, different genes are regulated by E2 during *in vivo* tumour growth compared to *in vitro* cell growth. Another array study linked E2-regulated changes in protein expression in human ovarian tumours to a predictive response of these patients to an aromatase inhibitor (Walker, MacLeod et al. 2007). Although that study examined a small number of proteins, there was some overlap with those identified in our gene expression study, such as *KRT7* (Walker, MacLeod et al. 2007). These results suggest that our mouse model could be used for

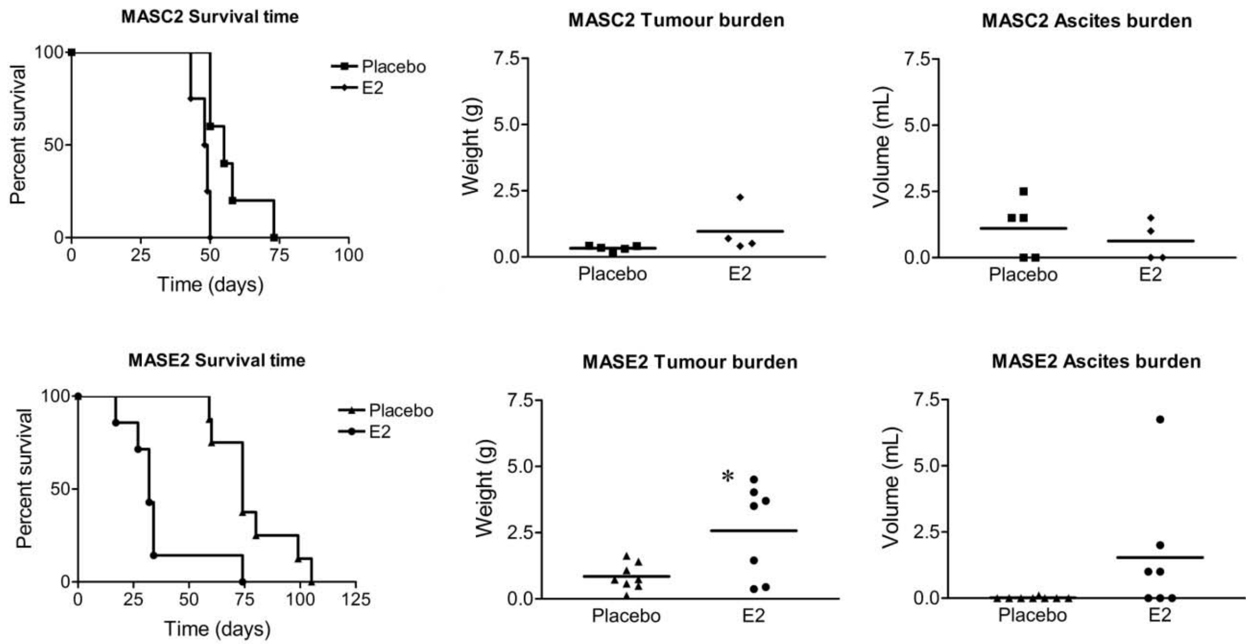
testing novel therapeutics and for the preclinical identification of genes that predict a response to endocrine therapy.

Perhaps most importantly, our microarray study uncovered novel genes regulated by E2 during ovarian cancer progression. Gene ontology analysis revealed that cell survival and proliferation, as well as the tumour microenvironment, are crucial for E2-stimulated growth of ovarian tumours *in vivo*. Interestingly, our gene array analysis revealed that *Greb1* is upregulated by E2 in mouse ovarian cancers. *Greb1* has previously been linked to breast and prostate cancer growth (Rae, Johnson et al. 2005; Rae, Johnson et al. 2006), but has never been examined in ovarian cancer. Although the function of *Greb1* is not known, the gene is induced following ERα binding to three estrogen response elements located upstream of the transcription start site (Deschenes, Bourdeau et al. 2007; Sun, Nawaz et al. 2007). Expression of *Greb1* is increased by exogenous E2 in breast cancer cells and ovarian cancer cells and following treatment with androgens in prostate cancer cells (Ghosh, Thompson et al. 2000; Rae, Johnson et al. 2005; Rae, Johnson et al. 2006; Deschenes, Bourdeau et al. 2007; Sun, Nawaz et al. 2007; Spillman, Manning et al. 2010). Suppression of *Greb1* via RNA interference inhibited the hormone-stimulated proliferation of both breast and prostate cancer cells (Rae, Johnson et al. 2005; Rae, Johnson et al. 2006). We found that *Greb1* is upregulated in mouse tumours and mouse ascites cell lines treated with E2 and is expressed in a sampling of all the major histological subtypes of human EOC (serous, endometrioid, clear cell and mucinous). Although the function of *Greb1* is still unknown, we propose that it plays an important role in hormone stimulated EOC cell survival and growth. The function of *Greb1* in ovarian cancer cells is currently under investigation in our lab.

In addition to *Greb1,* our microarray study identified other genes that were induced by exogenous E2 in mouse ovarian cancers, such as *Stc2*, *PgR* and *Cyp11a1*. *Stc2*, *PgR* and *Cyp11a1* are known to be E2-responsive genes (Graham, Roman et al. 1995; Charpentier, Bednarek et al. 2000; Bouras, Southey et al. 2002; Raulic, Ramos-Valdes et al. 2008; Allan, Couse et al. 2010; Kocanova, Kerr et al. 2010), but their ability to affect ovarian tumourigenesis has not been examined. *Stc2* is a glycoprotein hormone that is expressed in the ovary and is induced by E2 in ERα positive breast cancer cells (Honda, Kashiwagi et al. 1999; Charpentier, Bednarek et al. 2000; Bouras, Southey et al. 2002; Luo, Pisarska et al. 2005; Raulic, Ramos-Valdes et al. 2008). *PgR* is expressed in normal ovary and malignant EOCs (Janne, Kauppila et al. 1980; Galli, De Giovanni et al. 1981; Jones, Edwards et al. 1983; Willcocks, Toppila et al. 1983). *Cyp11a1* is found in the ovary and encodes for an enzyme responsible for the first step in the steroid hormone biosynthesis pathway, the conversion of cholesterol to pregnenolone (Miller 1988; Shi, Zhang et al. 2009). Each of these gene targets may be important mediators of hormone-regulated EOC initiation and progression and warrant further characterization.

We have shown that E2 is able to accelerate ovarian cancer progression *in vivo* despite little effect on *in vitro* proliferation of mouse ascites cell lines. Gene expression analysis of E2 treated ovarian tumours indicated that E2 affects a host of genes involved in cell proliferation, differentiation, and angiogenesis. Therefore the effects of E2 on ovarian cancer progression are likely mediated less through its mitogenic effects and more strongly through its ability to enhance cell migration, metastasis, survival and angiogenesis. Most importantly, we have identified specific genes involved in this process, such as *Greb1*, *Cyp11a1*, *Stc2*, *Cdh2*, *PgR* and *Kdr*. *Greb1* has previously been

shown to accelerate the growth of breast and prostate cancer, but has not been investigated in ovarian carcinomas. Herein we demonstrated that *Greb1* is induced by E2 in mouse ovarian tumours and is expressed in all of the histological subtypes of human EOC. We propose that *Greb1* is a novel mediator of ovarian cancer progression and may be used to predict the response to endocrine therapies in women suffering from EOC.



Supplemental Figure 1. The survival times and tumour burdens of ovariectomized mice grafted with mouse ascites cell lines and treated with E2 or a placebo. Immunodeficient mice grafted with MASC2 or MASE2 cells and treated with E2 have significantly decreased survival times compared to the placebo-treated controls (*, MASC2 survival curve, *P*=0.01, N=4-5 mice; MASE2 survival curve, *P*=0.003, N=7-8 mice, Logrank test). E2 treatment increased the tumour burden in ovariectomized mice grafted with MASE2 cells (*, *P*=0.02, *t* test, N=7-8 mice).

Chapter 4: Induction of a menopausal state alters the growth and histology of ovarian tumours in a mouse model of ovarian cancer

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Abstract

Objective: Ovarian cancer is often diagnosed in women after menopause when the levels of the serum gonadotropins follicle stimulating hormone (FSH) and luteinizing hormone (LH) are elevated due to the depletion of growing follicles within the ovary. The ability of FSH and LH to modulate the disease has not been well-studied due to a lack of physiologically relevant models of ovarian cancer. In this study, 4-vinylcyclohexene diepoxide (VCD) was used to deplete ovarian follicles and increase the levels of circulating FSH and LH in the tgCAG-LS-TAg mouse model of ovarian cancer.

Methods: VCD-induced follicle depletion was performed either before or after induction of the oncogene SV40 TAg in the ovarian surface epithelial cells of tgCAG-LS-TAg mice, which was mediated by the intrabursal delivery of adenovirus expressing Cre recombinase (AdCre).

Results: tgCAG-LS-TAg mice injected with AdCre developed undifferentiated ovarian tumours with mixed epithelial and stromal components and some features of sex cord stromal tumours. Treatment with VCD before or after AdCre injection yielded tumours of similar histology, but with the unique appearance of Sertoli cell nests. In mice treated with VCD prior to induction of tumourigenesis, the ovarian tumours tended to grow more slowly. The human ovarian cancer cell lines SKOV3 and OVCAR3 responded similarly to elevated levels of gonadotropins in a second model of menopause, growing more slowly in ovariectomized mice compared to cycling controls.

Conclusions: These results suggest that follicle depletion and elevated gonadotropins can alter the histology and the rate of growth of ovarian tumours.

Introduction

Ovarian cancer incidence increases during the peri- and postmenopausal years with 72% of ovarian cancer deaths occurring in women between the ages of 40 and 79 years (Jemal, Siegel et al. 2008). The reasons for this menopause-associated increase in incidence of ovarian cancer may include a depletion of ovarian follicles which alters the structural composition of the ovary, the associated reduction in ovarian and circulating estrogen levels, and the elevated serum levels of two gonadotropic hormones, follicle stimulating hormone (FSH) and luteinizing hormone (LH). The gonadotropin theory of ovarian carcinogenesis was first proposed by Cramer and Welch and suggests that the high levels of FSH and LH present during the menopausal years contribute to the risk of developing ovarian cancer by stimulating proliferation of the ovarian surface epithelium (OSE) which leads to malignant transformation (Cramer and Welch 1983). The OSE layer is believed to be the tissue of origin of most cases of epithelial ovarian cancer (Auersperg, Wong et al. 2001), although recent evidence suggests that the serous subtype of ovarian cancer may arise from the Fallopian tube epithelium (Medeiros, Muto et al. 2006; Kurman and Shih Ie).

Experiments performed in rodents support the theory that the hormonal changes associated with menopause (elevated gonadotropins and reduced estrogens and progestins) increase the risk of developing ovarian carcinogenesis. In adult rats and mice, there is little proliferation of OSE cells (<1%) under normal conditions; however, the administration of gonadotropins stimulated an eight- to ten-fold increase in OSE cell proliferation (Davies, Finnigan et al. 1999; Stewart, S. L., Querec et al. 2004a). Similarly, in a rat model of chemically-induced ovarian neoplasms, treatment with

gonadotropins increased the severity of the lesions in the OSE (Stewart, S. L., Querec et al. 2004b). While mimicking in animals the prolonged elevation in gonadotropin levels seen in menopause has resulted in ovarian tumourigenesis, the tumours do not appear to arise from the OSE. Transgenic mice generated to have chronic LH hypersecretion develop either granulosa cell tumours or luteomas, depending on the background strain (Risma, Clay et al. 1995; Keri, Lozada et al. 2000). FSH receptor knockout mice have high levels of circulating FSH and, by 12 months, more than 92% develop various kinds of ovarian pathology, including cysts and neoplasms of the sex cord stromal type (Danilovich, Roy et al. 2001). Thus, the prolonged elevation in levels of gonadotropins in the context of normal ovarian function appears to contribute to the development of ovarian cysts, granulosa cell tumours and stromal tumours but not OSE-derived neoplasms.

Human menopause occurs following the exhaustion of the oocyte pool which is not naturally modelled in mice, since mouse ovaries do not undergo a complete depletion of oocytes. Even mice at advanced ages continue to cycle and show only slight increases in circulating gonadotropins and decreases in estrogens and progesterone (Gee, Flurkey et al. 1983). Ovariectomized (OVX) animals are most widely used as models of menopause, and while they do undergo menopause-like hormonal changes (Gee, Flurkey et al. 1983) they cannot be used for studying ovarian tumour development. Recently a mouse model of menopause was developed which utilizes the industrial chemical 4-vinylcyclohexene diepoxide (VCD) to deplete the mouse ovaries of their follicles (Mayer, Devine et al. 2004). VCD targets the primordial and primary follicles, while the larger follicles remain unaffected. As a result, VCD-treated mice undergo a gradual decrease in

cyclicity and eventually enter persistent diestrus two months after treatment, resulting in elevated levels of FSH and LH, reduced levels of progesterone, and undetectable amounts of estradiol (consistent with human menopause) (Mayer, Devine et al. 2004).

Because of the hypothesized role of menopause in several pathological conditions, the VCD mouse model has been utilized to study atherosclerosis (Mayer, Dyer et al. 2005), insulin resistance (Romero-Aleshire, Diamond-Stanic et al. 2009), neuroprotection following stroke (Schauwecker, Wood et al. 2009) and ovarian tumourigenesis (Craig, Davis et al. 2010). When Craig and colleagues injected the carcinogen DMBA under the bursa of VCD-treated mouse ovaries (Craig, Davis et al. 2010), 57% developed ovarian neoplasms which were classified as Sertoli-Leydig tumours (a rare histological subtype of sex cord stromal tumours) (Craig, Davis et al. 2010). Previous studies have shown that rodents treated with DMBA develop thecal/granulosa cell tumours (Krarup 1970; Hilfrich 1975; Crist, Zhang et al. 2005), and it remains unclear whether the alterations in tumour histology resulted as a consequence of the differences in the follicular and hormonal environment, or due to the DMBA treatment which is a known genotoxic agent (Boutwell 1976; Ledwith, Joslyn et al. 1995; Marvanova, Vondracek et al. 2008).

Despite various attempts to model menopause and/or ovarian cancer, there are as yet no studies to determine the impact of follicle depletion and elevated serum LH and FSH on the rate of progression of cancers arising from the OSE. We recently reported the development of a transgenic model of ovarian cancer, tgCAG-LS-TAg, that utilizes the Cre/*loxP* system to inducibly express the oncogenic SV40 large and small T-Antigens (SV40 TAg) in the OSE. Following the delivery of adenovirus expressing Cre

recombinase (AdCre) under the ovarian bursal membrane, these mice develop ovarian tumours with a median survival of 113 days (Laviolette, Garson et al. 2010). Mice treated with 17β-estradiol have an earlier onset of tumourigenesis and a decreased survival time compared to placebo-treated controls (Laviolette, Garson et al. 2010). Because reproductive hormones can alter ovarian cancer progression in the tgCAG-LS-TAg mice (Laviolette, Garson et al. 2010), this study was designed to combine this mouse model of ovarian cancer with the VCD model of menopause to determine the effect of menopausal ovaries and elevated serum gonadotropins on ovarian tumour histology and progression.

Materials and Methods

tgCAG-LS-TAg Adenovirus Administration and VCD treatments

All animal experiments described in this study were performed according to the *Guide to the Care and Use of Experimental Animals* established by the Canadian Council on Animal Care. Mice were housed on a 12L:12D cycle, with free access to food and water. The generation and characterization of the transgenic tgCAG-LS-TAg mouse model of ovarian cancer was previously described (Laviolette, Garson et al. 2010). Delivery of recombinant adenoviruses Ad5CMVeGFP (AdGFP) or Ad5CMVCre (AdCre) (Vector Development Laboratory, Houston, TX, USA) to the OSE cells of tgCAG-LS-TAg mice (FVB/n background) was performed via intrabursal injection, as previously described (Clark-Knowles, Garson et al. 2007; Laviolette, Garson et al. 2010). tgCAG-LS-TAg mice were used in two studies. In the first study 6-8 week old mice (sexually mature (Vandenbergh 1967)) underwent intrabursal injection of AdCre (n= 10)

or AdGFP (n=11) and, after 15-20 days to recover from surgery, VCD treatments were initiated. Mice injected with AdCre alone (no VCD treatment) at 6-8 weeks of ages were used as controls (n=11). In the second study mice (5-6 weeks old) were first treated with VCD and once their ovaries were depleted of follicles (60 days) they were injected intrabursally with either AdCre (n=10) or AdGFP (n=10). The VCD treatment regimen has been previously described (Mayer, Devine et al. 2004), and consists of daily intraperitoneal injections with 160 mg/kg of VCD for 15 consecutive days. Day 1 was the first day of treatment and follicle depletion was complete by Day 60 (Mayer, Devine et al. 2004). Two mice from the first study were euthanized on Day 60 to confirm that the ovaries were depleted of follicles. The remaining mice were monitored for tumour development and euthanized when they reached a loss-of-wellness endpoint or after one year (for the AdGFP non-tumourigenic mice). The survival time reflects the length of time from the day of intrabursal injection of adenovirus until the day when the mice reached a loss-of-wellness endpoint with criteria including any one of the following: weight loss > 15 % of body weight, body weight increase > 5 g with respect to age-matched, non-tumourigenic controls, presence of abdominal distension that impairs mobility or affects wellness, respiratory distress, dehydration, anorexia, and/or diarrhea.

Plasma samples

Blood samples were collected from the mice via either the lateral saphenous vein or cardiac puncture into heparinized vials (Sarstedt, Germany). The plasma was separated via centrifugation (6000 rpm, for 10 minutes at 4°C) and stored at -20°C until use. The levels of FSH and LH were determined by RIA and sandwich IRMA by the

University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core.

Histologic Analysis and Immunohistochemistry

Tissues were collected from mice at necropsy, and the volume of ascites and weight of the total tumour burden were recorded. Tissues were fixed in 10% neutral-buffered formalin overnight, transferred to 70% ethanol, and paraffin-embedded. For histological analyses, 3-5 μm sections were cut and either stained with hemotoxylin and eosin (H&E) or used for immunohistochemistry (IHC). IHC was performed with each antibody, with at least 4 tissues per group, as previously described (Laviolette, Garson et al. 2010) to detect the following antigens: pan-cytokeratins (pan-CK; pre-diluted, Abcam); inhibinα (1:25, DAKO); calretinin (pre-diluted, Zymed); CK19 (Troma-3, 1:50, Developmental Studies Hybridoma Bank); estrogen receptor α (ERα; 1:50, Santa Cruz Biotechnology, Inc.); progesterone receptor (PgR; 1:50, LabVision); LHR (1:100, Santa Cruz Biotechnology, Inc.); and FSHR (1:500, Lifespan Biosciences). Rabbit Polymer HRP (DAKO) or rat secondary antibodies (1:200, DAKO) and streptavidin HRP (1:200, DAKO) were used prior to development with DAB. A mouse-on-mouse kit (Vector) was used according to the manufacturer's instructions to avoid cross-reactions when staining mouse tumours with mouse antibodies. The slides were counterstained with hematoxylin, dehydrated in a series of alcohols and xylenes, and coverslipped. Tissue sections were scanned using an Aperio ScanScope and images were captured using the Aperio ImageScope program (Aperio Technologies, Inc., Vista, CA, USA).

Ovarian Cancer Cell Xenografts

The human ovarian cancer cell lines SKOV3 (obtained from ATCC) and OVCAR3 (obtained from G. Mills; Houston, TX) were used in this study. The SKOV3 cells were grown in DMEM (Life Technologies, Burlington, Ontario, Canada) with 10% of a 1:3 mixture of fetal calf serum (FCS) and donor bovine serum (DBS) (CanSera, Rexdale, Ontario, Canada). The OVCAR3 cells were cultured in αMEM (Life Technologies, Burlington, Ontario, Canada) with 10% FCS-DBS. 10^7 SKOV3 or OVCAR3 cells were injected IP into normal (SKOV3 n=5 mice; OVCAR3 n=6 mice) or ovariectomized (OVX; SKOV3 n=6 mice; OVCAR3 n=6 mice) CD1 nude mice (Charles Rivers Laboratories, Montreal, QC). The mice were euthanized when they reached a loss-of-wellness endpoint (as described above) or after 150 days (end of study).

Statistical analyses

All data were plotted using GraphPad Prism software (version 3.02: GraphPad Software, San Diego, CA, USA). Kaplan-Meier survival curves were compared using a Logrank test. The hormone levels, tumour weights, and ascites volumes of tgCAG-LS-TAg mice treated with or without VCD were compared using *t* tests, except for the FSH plasma levels which were compared using a one-way ANOVA test and a Tukey's post-hoc test to identify statistical differences. Statistical significance was inferred at $P < 0.05$.

Results

VCD treatment of tgCAG-LS-TAg mice results in ovaries depleted of follicles

In the first study, half of the 6-8 week old tgCAG-LS-TAg mice (n=11) were injected with AdGFP under the ovarian bursa and then were treated with VCD to deplete the ovaries of follicles. tgCAG-LS-TAg mice injected with AdGFP do not develop tumours because Cre recombinase is required for induction of the SV40 TAg oncogene in this mouse model. Two mice treated with AdGFP then VCD were euthanized 60 days after the first dose of VCD to confirm that the ovaries were devoid of follicles. Although there were a few remnant granulosa cells from primordial and primary follicles present (confirmed by inhibinα staining), no growing follicles or oocytes could be found within the ovary (data not shown). The remaining mice (n=9) survived for one year after intrabursal injection of AdGFP without any evidence of ovarian tumours. Histological examination of ovarian sections revealed much smaller ovaries than age-matched, normally cycling (no VCD) controls (Figure 1). Moreover, the ovaries did not contain any growing follicles, despite the fact that the ovary is well perfused (several blood vessels can be seen in the H&E sections). The ovaries from VCD-treated mice did display areas of putative preneoplastic lesions on the OSE, including areas of hyperplasia, papillomatosis, invaginations, and small inclusion cysts.

IHC staining was used to determine the cell types that remained in the ovaries after VCD-induced follicle depletion. CK19 immunoreactivity was present in the OSE cells and in epithelial invaginations and inclusion cysts which were prevalent in the ovaries from VCD-treated mice (Figure 1B, close up in VCD then AdGFP panel).

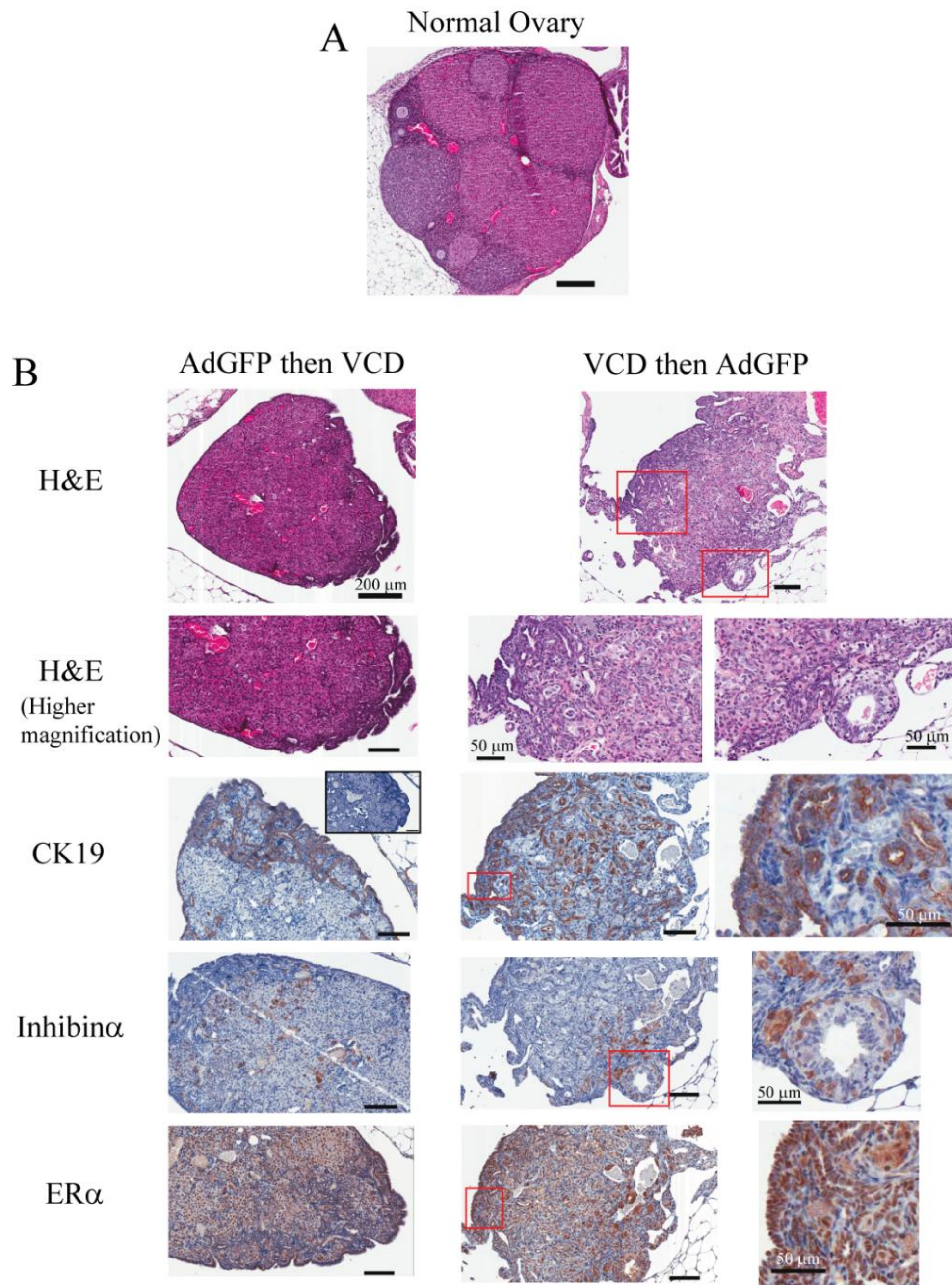

Figure 1. The histomorphology of mouse ovaries treated with VCD. A) H&E section of an ovary from a normally cycling mouse showing follicles and corpora lutea in different stages of growth. B) H&E sections of representative mouse ovaries injected intrabursally with AdGFP and treated with VCD. One year after VCD treatment, the ovaries show complete depletion of growing follicles. The ovary is composed mostly of luteinized stroma, but IHC for CK19 demonstrates an intact OSE as well as migration of epithelial cells into the ovarian stroma. Inhibinα positive cells highlight a few residual, non-growing follicles within the mouse ovaries. Both the OSE and stromal cells are strongly positive for ERα. The inset is a control processed with no primary antibody. The red boxes indicate areas that are shown in the higher magnification images. Scale bars represent 100 µm, unless otherwise indicated.

Inhibinα was localized to a few remaining granulosa cells from primordial and primary follicles, but those follicles did not contain oocytes. ERα was strongly expressed by the OSE layer and throughout the ovary (Figure 1B). The cellular morphology and immunophenotype of the ovaries from VCD-treated mice suggest that they are mainly composed of activated luteinized stromal cells and inclusions of epithelial cells, with few remaining granulosa or interstitial cells.

In the second study, half of the tgCAG-LS-TAg mice (n=10) were treated with VCD prior to the intrabursal injection of AdGFP (VCD then AdGFP), and as expected, the histology after one year was similar to mice treated with AdGFP then VCD (Figure 1B). These ovaries also contained CK19 positive epithelial cell invaginations and inclusion cysts, inhibinα positive granulosa cells in degenerate primordial and primary follicles, and were strongly positive for ERα throughout the ovary (Figure 1B).

In addition to the histological evidence to suggest menopause-like ovaries, the VCD treatment was sufficient to induce menopause-like changes in gonadotropic hormones. tgCAG-LS-TAg mice intrabursally injected with AdGFP and then treated with VCD had more than 7-fold higher levels of circulating FSH and greater than 50-fold higher levels of LH compared to normally cycling female mice (Figure 2). The plasma concentration of FSH was 8.73 ng/mL (n=4, Figure 2A) and the concentration of LH was 0.358 ng/mL (n=4, Figure 2B) in normally cycling mice, while the levels of FSH and LH in AdGFP then VCD-treated "menopausal" mice were 64.01 ng/mL (n=3, $P < 0.001$) and 18.26 ng/mL (n=3, $P = 0.0001$), respectively.

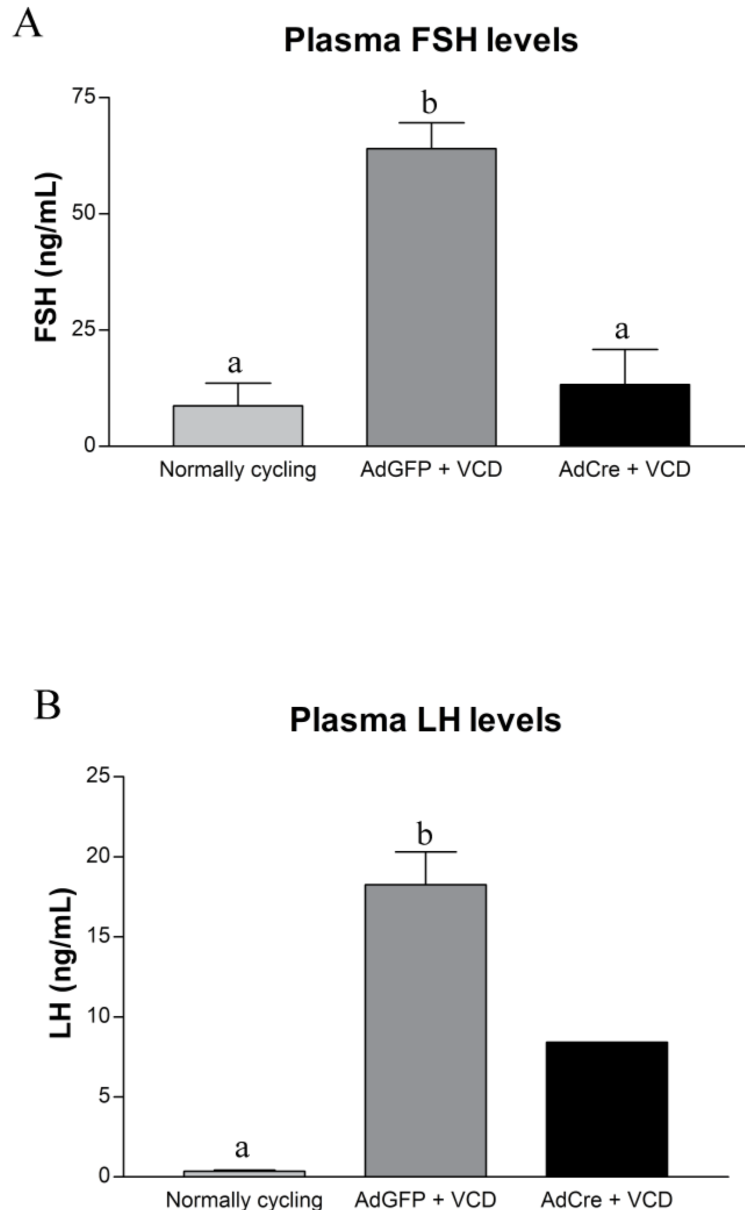

Figure 2. Circulating levels of LH and FSH following treatment with VCD. tgCAG-LS-TAg mice injected intrabursally with AdGFP and then treated with VCD had significantly higher circulating levels of FSH (A) and LH (B) compared to normally cycling female mice. A) Mice induced to form ovarian tumours and simultaneously treated with VCD (AdCre then VCD) had plasma FSH levels (13.27 ng/mL; n=2) similar to normally cycling females (8.73 ng/mL; n=4). The plasma samples were collected from mice at least 60 days after the first dose of VCD, once the mice were "menopausal." *a* is statistically different from *b* ((A) One-way ANOVA, $P < 0.001$ or (B) *t* test, $P = 0.0001$); Data are mean +/- standard error of the mean.

VCD treatment alters the growth and histomorphology of ovarian tumours

The control tgCAG-LS-TAg mice treated with AdGFP either before or after VCD did not develop ovarian tumours, despite elevated serum levels of FSH and LH and follicle depleted ovaries, indicating that VCD treatment alone does not result in ovarian cancer. To determine the effect of elevated gonadotropins and follicle depletion on ovarian tumour progression, tgCAG-LS-TAg tumourigenic mice (intrabursally injected with AdCre) were treated with VCD. In the first study, half of the mice (n=10) were injected intrabursally with AdCre (to induce tumourigenesis) and then treated with VCD to stimulate the transition to a menopause-like hormonal state during tumour progression (AdCre then VCD mice). In the second study, half of the mice (n=10) were treated with VCD and after follicle depletion (Day 60), were then intrabursally injected with AdCre to initiate tumourigenesis in the menopausal ovaries (VCD then AdCre mice). Mice treated with AdCre alone (no VCD) were used as controls, and it has already been shown that tgCAG-LS-TAg mice injected with AdGFP (without VCD) survive for up to one year without any evidence of ovarian tumours (Laviolette, Garson et al. 2010).

The AdCre then VCD treated mice developed ovarian tumours with a median survival time of 120 days (n=10) which was not statistically different from the control mice treated with AdCre alone (survival time of 113 days, n=11; Figure 3A). Similarly, there were no statistical differences in total tumour burden or ascites volumes in mice treated with AdCre alone (mean tumour burden, 4.3 g; mean ascites volume, 2.17 mL) vs. the AdCre then VCD mice (mean tumour burden, 3.24 g; mean ascites volume 1.24 mL, Figure 3A). The tgCAG-LS-TAg mice that were treated with VCD (n=10) prior to

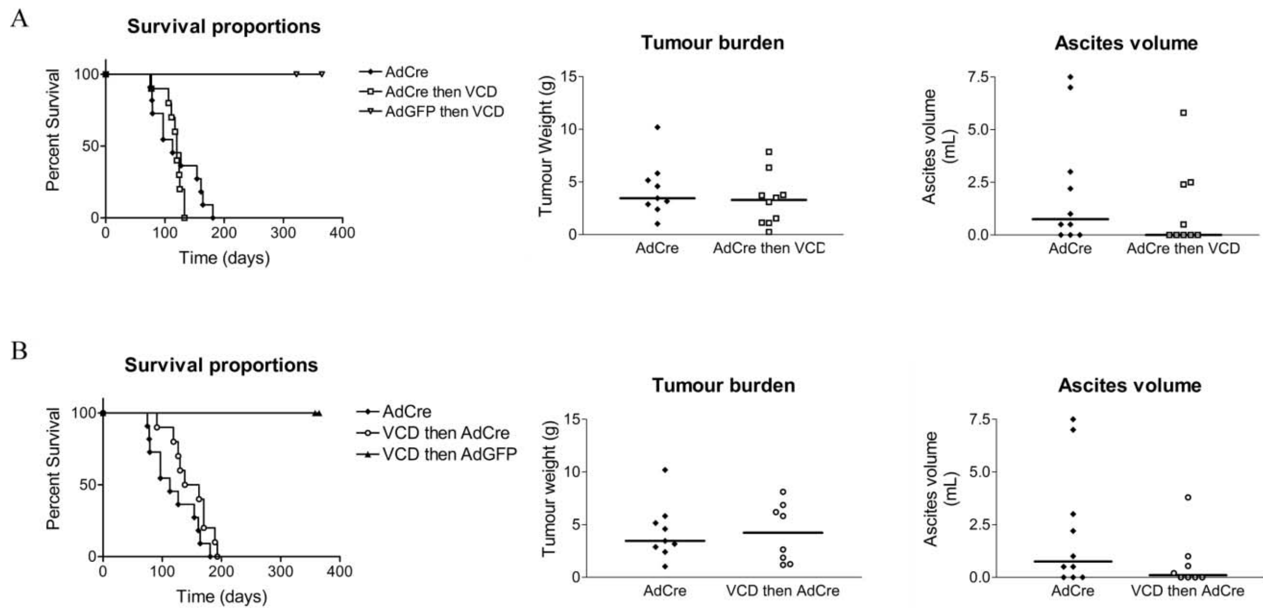

Figure 3. The Kaplan-Meier survival curves, total tumour burden and ascites volumes of tgCAG-LS-TAg mice injected intrabursally with AdCre and treated with VCD. A) tgCAG-LS-TAg mice injected with AdCre alone developed ovarian tumours with a median survival time of 113 days (AdCre, n=11). tgCAG-LS-TAg mice injected with AdCre and then treated with VCD survived a median of 120 days (AdCre then VCD, n=10). B) tgCAG-LS-TAg mice treated with VCD followed 60 days later by intrabursal injection of AdCre survived a median of 150 days (VCD then AdCre, n=10) which showed a trend towards a slower rate of tumour progression compared with the mice receiving only AdCre (AdCre, n=11; Logrank test, $P = 0.07$). There were no differences in tumour burden or ascites volume when comparing controls (AdCre alone) to mice treated with both AdCre and VCD (either AdCre then VCD (A) or VCD then AdCre (B)). The lines represent the median tumour weights and ascites volumes for each group. The control non-tumourigenic tgCAG-LS-TAg mice (AdGFP then VCD, n=9 and VCD then AdGFP, n=10) were euthanized one year after injection of AdGFP without any evidence of ovarian tumours.

induction of the SV40 TAg oncogene tended to survive longer than the AdCre alone (n=11) controls (150 days vs. 113 days, $P = 0.07$) but this difference did not reach statistical significance (Figure 3B). There were no differences in tumour burden or ascites volume between these two groups at disease endpoint (VCD then AdCre – mean tumour burden, 4.24 g; mean ascites volume, 0.69 mL, Figure 3B). Although not significantly different, the incidence of ascites in both studies was notably reduced from 70% of control (AdCre alone) mice to 44-50% of mice receiving both VCD and AdCre treatments (Figure 3).

A panel of IHC markers was used to determine the histology of the ovarian tumours from mice treated with AdCre and VCD. Previously we reported that the ovarian tumours in tgCAG-LS-TAg mice injected with AdCre are poorly differentiated, high grade tumours with both epithelial and stromal components and some characteristics of sex cord stromal tumours (Laviolette, Garson et al. 2010). In this study we analyzed four randomly selected tumours from each of the VCD treatment groups (AdCre then VCD and VCD then AdCre). The tumours in mice treated with both VCD and AdCre were undifferentiated malignant ovarian cancers with a high mitotic grade (greater than 10 mitoses per high powered field of view as determined by a pathologist, MKS), and like the tumours obtained from mice treated with AdCre alone, displayed areas of insular formations and cords suggestive of sex cord stromal tumours (Figure 4). A notable distinction in both groups of VCD and AdCre treated mice, compared with the mice treated with AdCre alone, was the appearance of Sertoli nests (Figure 4, arrows) which are a histological characteristic of Sertoli tumours, and which were not found in the

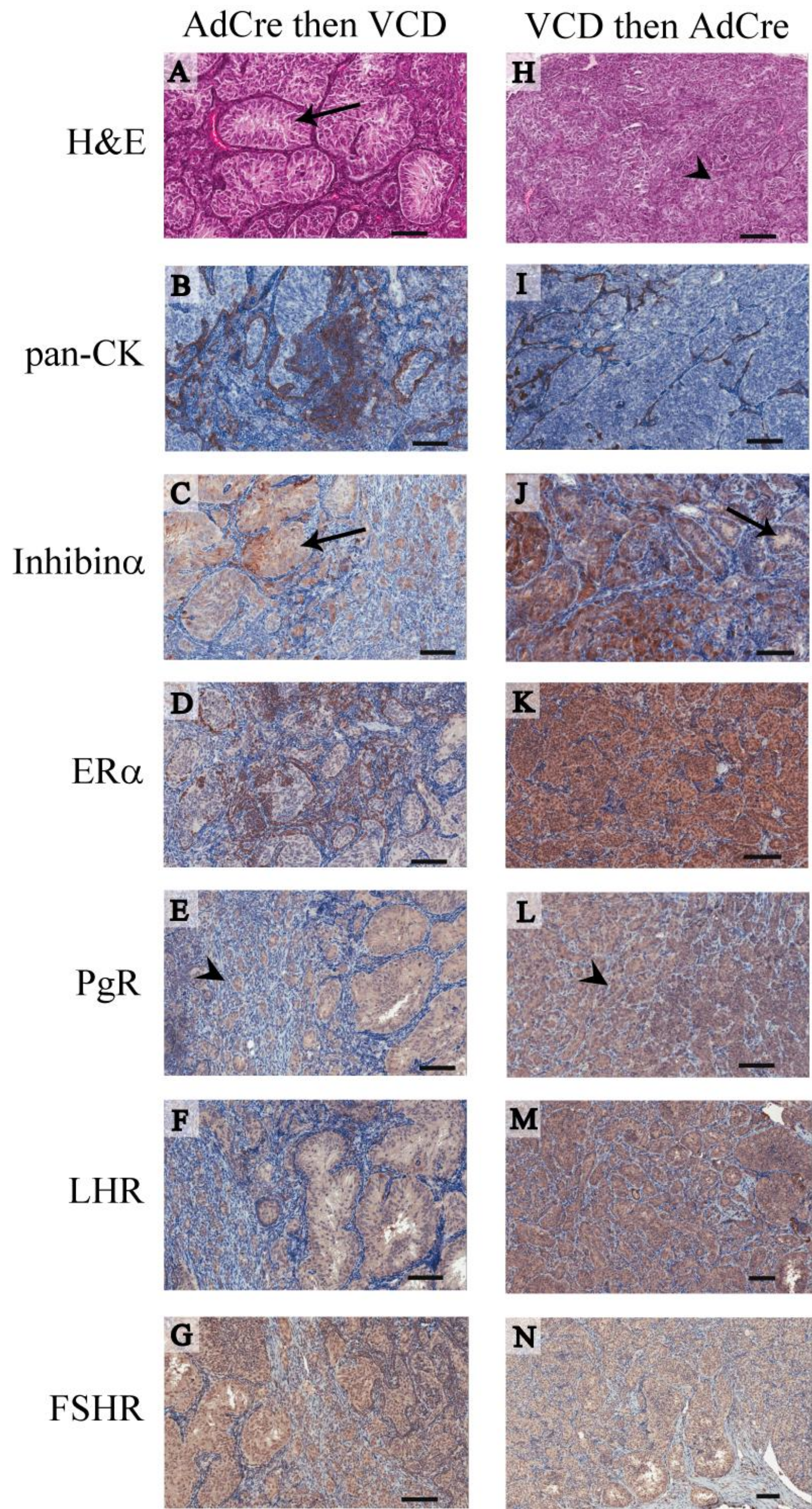

Figure 4. The histological profile of mouse ovarian tumours from tgCAG-LS-TAg mice treated with VCD. The tumours from mice intrabursally injected with AdCre before or after treatment with VCD have histological characteristics of both Sertoli cell tumours, including nests of inhibinα positive Sertoli cells (arrows; A, C, J), and granulosa cell tumours, including areas of cysts and insular formations (arrowheads; E, H, L). The tumours were focally positive for both cytokeratins (B, I) and inhibinα (C, J), and expressed high levels of ERα (D, K), PgR (E, L), LHR (F, M) and FSHR (G, N).

tumours from mice injected with AdCre only (without VCD). Moreover, the tumours from mice treated with both VCD and AdCre were mixed containing characteristics of both granulosa cell tumours (Figure 4, arrowheads) and Sertoli tumours (Figure 4, arrows) which are two histological subtypes of sex cord stromal tumours.

The IHC characterization of the ovarian neoplasms demonstrated that tumours from both of the VCD treated groups were mixed in histology. They were positive for inhibinα and focally positive for calretinin and vimentin, further suggesting a sex cord stromal phenotype, but they also contained epithelial cells as indicated by the presence of cytokeratin expressing cells (Figure 4). The tumours obtained from the control mice (AdCre alone) similarly focally expressed inhibinα and pan-cytokeratin, despite their poorly differentiated histology (previously reported in (Laviolette, Garson et al. 2010)).

All of the tumours arising in VCD-treated mice stained strongly for the steroid hormone receptors ERα and PgR and the gonadotropin hormone receptors LHR and FSHR. Although the sample size was small, the plasma levels of FSH and LH were decreased in the mice treated with AdCre then VCD (compared to AdGFP then VCD mice; Figure 2), suggesting that the tumours may be producing steroid hormones (a characteristic of Sertoli and granulosa cell tumours (Young 2005; Pectasides, Pectasides et al. 2008)), which could negatively feed back to the pituitary to decrease the levels of circulating gonadotropins.

Xenografted human ovarian cancer cell lines grow more slowly in ovariectomized mice

To examine whether elevated gonadotropin levels could alter the growth of human ovarian cancer cells, OVCAR3 and SKOV3 cells were xenografted into OVX mice, as a second model of menopause. With both cell lines, the tumours grew slower in OVX mice compared to mice with intact ovaries (Figure 5). OVX mice xenografted with the OVCAR3 cells had a significantly increased median survival time compared to normally cycling controls (111 days vs. 85 days respectively, n=6 mice per group, P = 0.03). Moreover, two of the OVX mice (33%) xenografted with OVCAR3 cells did not have tumours when the mice were euthanized at the end of the study (150 days). Similarly, SKOV3 cells did not form tumours as frequently in OVX mice (n=6) as in the controls (n=5). At the end of the study (150 days), one control mouse (20%) and four OVX mice (67%) did not have tumours. These results suggest that elevated levels of gonadotropins may inhibit ovarian cancer cell growth.

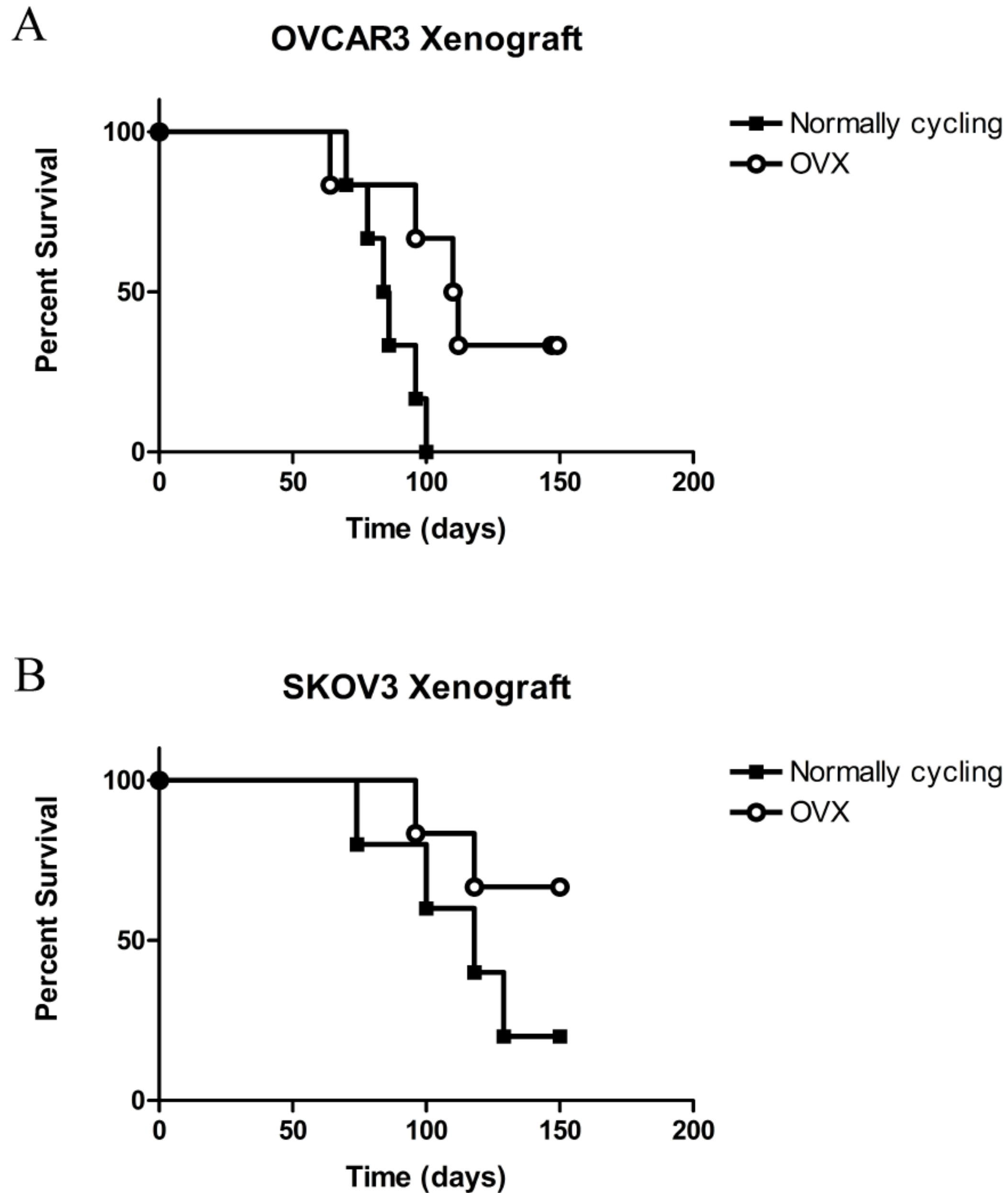

Figure 5. Survival time of OVX mice xenografted with human ovarian cancer cell lines. A) OVX nude mice xenografted with the human ovarian cancer cell line OVCAR3 survived significantly longer (median of 111 days, n=6 mice) than did normally cycling controls (ovaries intact), which survived a median of 85 days (n=6 mice; Logrank test, *P*=0.03). B) 80% of normally cycling mice xenografted with SKOV3 cells developed tumours (median survival time of 118 days; n=5 mice), whereas only 33% of the OVX mice developed tumours in the same period (n=6; end of study was 150 days).

Discussion

The loss of ovarian function during menopause and the associated increase in the serum gonadotropins has long been hypothesized to increase the risk of developing ovarian cancer, as evidenced by the increase in the incidence and mortality in women during their menopausal years (Jemal, Siegel et al. 2008). The ability to study the effects of elevated gonadotropins on ovarian tumourigenesis has been precluded by a lack of animal models of ovarian cancer in which tumours arise from the epithelium of the ovary with controlled timing of onset of tumour initiation and a rate of tumour progression that could be influenced by hormones. In this study, we describe the first combination of a mouse model of menopause with a transgenic mouse model of ovarian cancer. The results show that induction of a menopause-like state (ovarian follicle depletion and elevated levels of serum gonadotropins) modestly prolonged survival in the tgCAG-LS-TAg model of ovarian cancer and altered the histological phenotype of the resulting tumours.

There were several advantages to using the tgCAG-LS-TAg mouse model of ovarian cancer in this study, including the controlled spatial and temporal initiation of tumourigenesis in the OSE cells, the long latency (approximately 80 days) from the time of SV40 TAg induction in the OSE until tumours are initiated, and the previous demonstration that this model responds to the reproductive hormone 17β-estradiol, suggesting that disease progression in this model can be modulated by reproductive hormones (Laviolette, Garson et al. 2010). Most importantly, the tumours develop *in situ*, which one predicts would have greater clinical relevance than xenograft models of this disease.

The tgCAG-LS-TAg mice injected intrabursally with AdCre alone develop ovarian tumours with a median overall survival time of 113 days. The poorly differentiated, highly aggressive ovarian tumours contain both epithelial and stromal cells and display characteristics of sex cord stromal tumours (Laviolette, Garson et al. 2010). The slower progression of ovarian tumour growth in the tgCAG-LS-TAg mice treated with VCD and AdCre (compared to AdCre alone) could be due to a number of factors. There may be lingering toxic effects of VCD on the somatic cells that do not affect the ability of OSE to be transformed by SV40 TAg, but that might affect their rate of proliferation. Alternatively, the effects of VCD could be due to its ability to eliminate the follicles, rendering the ovaries much smaller than ovaries of normally cycling mice, and therefore the surrounding OSE layer consists of fewer cells. Since the induction of tumourigenesis in the tgCAG-LS-TAg mouse model is dependent on successful adenoviral infection of the OSE cells, a reduction in the total number of OSE cells may reduce the efficiency of adenoviral infection, Cre-mediated recombination and in turn SV40 TAg driven transformation of the OSE cells. Moreover, the VCD treatments were initiated shortly after the tgCAG-LS-TAg mice become reproductively mature, and as a result the mice underwent few ovulations before VCD depleted the ovaries of their follicles (at approximately three to four months of age). Ovulations are a risk factor for ovarian cancer (Fathalla 1971; Casagrande, Louie et al. 1979) and therefore the reduced number of ovulations prior to the initiation of ovarian tumours may contribute to the trend toward an increased survival time in the tgCAG-LS-TAg mice treated with VCD then AdCre. It would be interesting to determine if VCD-mediated follicle depletion and elevated gonadotropins could accelerate ovarian cancer progression in older female

tgCAG-LS-TAg mice that have undergone many ovulatory cycles prior to tumour initiation.

It is also possible that the increased levels of serum LH and FSH protect the OSE cells from transformation. It has previously been shown that combinations of FSH and human chorionic gonadotropin (a hormone similar to LH) decrease ovarian cancer cell proliferation (Tourgeman, Lu et al. 2002). Similarly, FSH has been shown to decrease proliferation of OSE cells collected from the ovaries of postmenopausal women (Ivarsson, Sundfeldt et al. 2001). Attempts in our lab to harvest and grow OSE cells from mice treated with VCD were unsuccessful, despite the establishment of several primary OSE cultures from untreated normal mice after superovulation (unpublished data), which suggests that the cells from VCD treated mice take on a more quiescent phenotype. Whether this alteration in the growth potential of OSE cells is due to the direct effects of VCD itself or to the physiological consequences of follicle depletion and elevated FSH and LH remains unclear. However, OSE cells that are quiescent are less likely to undergo transformation, even following induction of SV40 TAg. Moreover, the elevated levels of LH and FSH could protect the OSE cells *in vivo* by decreasing the serum levels of steroid hormones. We have previously shown that 17β-estradiol can accelerate the initiation of ovarian tumourigenesis in this mouse model (Laviolette, Garson et al. 2010) and it has been shown that VCD treatment decreases circulating steroid levels (Mayer, Devine et al. 2004). Therefore the increased survival in the VCD then AdCre tgCAG-LS-TAg tumourigenic mice might also be due to a decrease in circulating 17β-estradiol.

An interesting observation in this study was the ability of VCD treatment to alter the histology of ovarian tumours in the tgCAG-LS-TAg mouse model, which may also

explain the trend for increased survival in these mice. tgCAG-LS-TAg mice (injected with AdCre alone) develop poorly differentiated, aggressive tumours, whereas the mice treated with VCD and AdCre develop more differentiated ovarian tumours displaying characteristics of both Sertoli tumours and granulosa cell tumours. Sertoli tumours and granulosa cell tumours are histological subtypes of the sex cord stromal ovarian cancers, which account for approximately 5-7% of ovarian malignancies in women (Chen, V. W., Ruiz et al. 2003; Zhang, Cheung et al. 2007). Women with stage III-IV sex cord stromal cancers have a 5-year survival rate of 45.5%, compared with 29.1% for women diagnosed with epithelial cancers at the same stage (Chan, Cheung et al. 2006). Sex cord stromal cancers are most frequently diagnosed in women during the peri-menopausal or early post-menopausal years (Chen, V. W., Ruiz et al. 2003; Zhang, Cheung et al. 2007). Generally the stage, grade and histology of ovarian tumours predict the 5-year survival of patients, but younger women (≤ 50 years) diagnosed with sex cord stromal tumours have a better 5-year survival compared to older women (> 50 years), further emphasizing the importance of the post-menopausal state in the progression of this histological subtype of ovarian tumours (Zhang, Cheung et al. 2007).

The transition in the histological subtype from undifferentiated ovarian tumours to Sertoli and granulosa cell tumours in the tgCAG-LS-TAg mice could be as a result of elevated gonadotropins following VCD exposure or due to the loss of growing follicles, but is likely due to germ cell loss since it has previously been reported that ovaries depleted of follicles can revert to tissue resembling testis (Behringer, Cate et al. 1990; Couse, Hewitt et al. 1999). The sequence of events following oocyte/follicle dissolution generally includes the appearance of hypertrophied stromal cells (likely due to elevated

gonadotropin stimulation), proliferation of the OSE and invagination into the ovarian stroma. Depending on the model, the subsequent events can include granulosa cell transdifferentiation into Sertoli cells that form empty testis-like tubules (Stoica, Koestner et al. 1985). However, the tubular structures arising from the ingrowth of surface epithelial cells can also assume similar testis-like features (Liebelt, Sass et al. 1987). Since the ovaries from tgCAG-LS-TAg mice treated with VCD and AdGFP contained very few granulosa cells, but did contain many CK19 positive inclusion cysts it is reasonable to speculate that in this model the ovarian tumours originated from OSE cells that transdifferentiated into Sertoli-like cells.

A recent study has shown that VCD in combination with the direct application of the carcinogen DMBA to the ovary can increase the penetrance of ovarian tumours and shift the phenotype from thecal/granulosa tumours to Sertoli-Leydig cell tumours (Craig, Davis et al. 2010). While the use of a genetic model of ovarian cancer rather than chemically-induced tumours might be considered a refinement, it is interesting to note that both models of ovarian carcinogenesis result in tumours with Sertoli cell components, despite both models generating different tumour types in the absence of VCD. We demonstrated that VCD treatments in the tgCAG-LS-TAg model of ovarian cancer can modify the histology of the disease from poorly differentiated tumours to Sertoli tumours, likely through a reversion of the VCD-treated ovary to a tissue phenotypically resembling testis.

In this study we have shown that VCD treatment depletes mouse ovaries of their follicles, and in turn alters the hormonal milieu by increasing serum gonadotropins. Similar to human ovarian histology after menopause (Okamura and Katabuchi 2001), the

VCD and AdGFP-treated "menopausal" mice have an increase in ERα positive epithelial inclusion cysts, structures which are believed to be potential ovarian cancer precursors. A recent study showed that inclusion cysts in human post-menopausal ovaries, as well as the OSE cells, stain positive for ERα expression (Brodowska, Laszczynska et al. 2007). Despite the appearance of OSE cell hyperplasia and inclusion cysts within the VCD and AdGFP treated ovaries, the menopause-like state was not sufficient for malignant transformation of the OSE, possibly due to a protective effect of elevated gonadotropins or loss of steroid hormone growth support. When the VCD model of menopause was combined with the tgCAG-LS-TAg mouse model of ovarian cancer, the resulting tumours grew slower than in mice not treated with VCD, implying a protective effect of the gonadotropins. Moreover ovarian tumours from tgCAG-LS-TAg mice treated with VCD had an altered histological appearance compared to tumours from mice not treated with VCD. Although using mice with oocyte-depleted ovaries is more appropriate for modelling human menopause, we also used a second mouse model of menopause, the OVX model. We have shown in the OVX model that the hormonal changes associated with menopause (elevated gonadotropins, decreased steroid hormones) can also slow the growth of human ovarian cancer cells. Immunodeficient OVX mice xenografted with the human ovarian cancer cell lines OVCAR3 or SKOV3 had an increased survival time and a decreased incidence of tumour formation compared to control mice.

Conclusions

In this study, we have shown that menopausal state (follicle depletion, elevated gonadotropins and reduced steroid hormones) can alter both the rate of tumour

progression and the histology of ovarian cancer. Taken together these results indicate that menopause plays an important role in ovarian tumourigenesis and that the use of hormone replacement therapy (exogenous estrogens and progestins) may have a double impact on promoting ovarian tumour progression by increasing estrogens and decreasing gonadotropins.

Chapter 5: Discussion

Summary of findings

The aim of this PhD research project was to evaluate the role of the reproductive hormones, specifically E2, P4, LH, and FSH, on ovarian cancer initiation and progression, employing a well-characterized murine model that I developed for this study. We have generated a transgenic mouse model, tgCAG-LS-TAg, which relied on the Cre/*loxP* system to inducibly express the potent oncogene SV40 TAg in the OSE. Following Cre mediated recombination, mice developed poorly differentiated ovarian tumours with a latency of approximately 90 days. The mice had an overall median survival time of 113 days, which was not significantly affected by exogenous P4. Interestingly, tgCAG-LS-TAg mice treated with E2 had a significantly shortened median survival time of only 50 days. The decreased survival was due to an earlier onset of ovarian tumourigenesis, most likely because E2 treatment increased the number of putative preneoplastic lesions in the OSE. Moreover, mouse ascites cell lines derived from the tgCAG-LS-TAg mice retained their responsiveness to E2. When these cells were grafted into immunodeficient mice, E2 treatment significantly accelerated disease progression, which could be the result of increased expression of the E2 responsive gene, *Greb1*. These results further support the hypothesis that E2 can affect both ovarian cancer initiation and progression.

The role of menopause in ovarian cancer progression was examined by using VCD to gradually deplete tgCAG-LS-TAg mice of their oocytes. We found that VCD-

induced menopause prolonged the survival time of tumourigenic tgCAG-LS-TAg mice, perhaps in part due to a shift in the histological subtype from a poorly differentiated ovarian tumour to a Sertoli tumour in the VCD-treated mice. A second model of menopause, the OVX model, was employed to study ovarian cancer progression in the absence of ovarian function. We found that even in the absence of residual ovarian tissue, the hormonal changes associated with menopause were sufficient to prolong disease progression because OVX mice xenografted with ovarian cancer cells had fewer tumours and longer overall survival times compared to mice with intact ovaries. Taken together, these results confirm the importance of the reproductive hormones on ovarian cancer etiology and highlight the value of representative disease models for the development of prevention and treatment strategies.

General Discussion

When designing transgenic mouse models of cancer, one of the most useful tools is a tissue specific promoter to activate oncogenes or inactivate tumour suppressor genes precisely in the cell type where the human cancers originate. Several mouse models of prostate, breast and pancreatic cancer have been successfully created using tissue specific promoters (Stewart, T. A., Pattengale et al. 1984; Quaife, Pinkert et al. 1987; Bouchard, Lamarre et al. 1989; Greenberg, DeMayo et al. 1995; Yoshidome, Shibata et al. 1998). Generating mouse models of ovarian cancer is challenging because at this time there is no known promoter that drives gene expression uniquely in the OSE cells. To circumvent

this problem, transgenic models of ovarian cancer generated in the past relied on promoters expressed throughout the reproductive tract, including the OSE, to drive expression of oncogenes. Although these models resulted in ovarian tumours, the tumours appeared embryonically and in other reproductive tissues, as well as in the OSE (Connolly, Bao et al. 2003; Garson, Macdonald et al. 2003).

In order to direct gene expression to the OSE cells specifically, we and others have utilized the bursal membrane which surrounds rodent ovaries. Adenoviruses expressing Cre recombinase (AdCre) are intrabursally injected to inactivate floxed tumour suppressor genes or activate oncogenes in the OSE (Flesken-Nikitin, Choi et al. 2003; Dinulescu, Ince et al. 2005; Clark-Knowles, Garson et al. 2007; Wu, Hendrix-Lucas et al. 2007; Clark-Knowles, Senterman et al. 2009; Laviolette, Garson et al. 2010). We have generated a model which employs the Cre/*loxP* system to conditionally activate the oncogene SV40 TAg after intrabursal delivery of AdCre (Laviolette, Garson et al. 2010). The benefits of our model over pre-existing models are: 1) the tumours arise from the OSE cells; 2) there is a long latency from the time of SV40 TAg induction (between 7-14 days after AdCre) until the appearance of latent tumours (approximately 90 days after AdCre); and 3) the progression mimics human disease with peritoneal ascites and metastatic spread throughout the abdomen (favouring the mesentery lining of the diaphragm, omentum, intestines, and peritoneum). Moreover, the model responds to reproductive hormones, specifically the gonadotropins (Laviolette, Ethier et al. 2011) and E2 (Laviolette, Garson et al. 2010), implying its usefulness for evaluating other disease modulators and therapeutics. Because of the advantages of the tgCAG-LS-TAg mouse model, it is already being used in collaboration with researchers at the Kolling Institute of

Medical Research in Australia for the identification of serum biomarkers and the evaluation of novel treatments for ovarian cancer.

Although there are many advantages to the tgCAG-LS-TAg model of ovarian cancer, there are some limitations. The major limitations are: 1) the microsurgeries that are required to activate SV40 TAg; and 2) the fact that the poorly differentiated histology in the resulting ovarian tumours is not representative of the most common histological subtype in humans, the serous carcinomas. The other transgenic mouse models of EOC develop either endometrioid or poorly differentiated carcinomas, but display overlapping characteristics with the serous histological subtype, such as cytokeratin positivity throughout the tumours (Connolly, Bao et al. 2003; Flesken-Nikitin, Choi et al. 2003; Dinulescu, Ince et al. 2005; Wu, Hendrix-Lucas et al. 2007). Our model exhibits only focal expression of the cytokeratins by immunohistochemistry. We can speculate that this may due to intrinsic differences between mouse strains, since experiments in our lab using the same gene targeting vectors to express SV40 TAg in the OSE result in more epithelial tumours (cytokeratin positive) in mice on the C57Bl/6 compared to the FVB/n background (Vanderhyden lab, unpublished data). All of the previously derived transgenic mouse models of EOC have at least a percentage of the C57Bl/6 strain, although most are on mixed backgrounds (Connolly, Bao et al. 2003; Flesken-Nikitin, Choi et al. 2003; Dinulescu, Ince et al. 2005; Wu, Hendrix-Lucas et al. 2007). Since the tgCAG-LS-TAg mice were created on the homozygous FVB/n background, we can speculate that their susceptibility for poorly differentiated tumours is due, at least in part, to their strain. Perhaps changing the genetic background of these mice would be sufficient for generating ovarian tumours of the serous histological subtype.

Recently is has been suggested that some serous ovarian carcinomas, mainly those arising in women with BRCA1 germline mutations, may originate from the fimbria of the fallopian tube (Crum, Drapkin et al. 2007). Interestingly, there are currently no transgenic mouse models where tumourigenesis is initiated specifically in the oviduct, which is the mouse equivalent to the human fallopian tube. It is anticipated that these mouse models will be created soon and will be very valuable for delineating the role of the OSE cells vs. the fallopian tube epithelium in the development of serous EOCs. A better understanding of ovarian cancer initiation, including the tissue(s) of origin, is required for the development of more effective screening and early detection methods.

We have compelling evidence to suggest that the reproductive steroid hormone E2 significantly accelerates ovarian cancer progression. This was shown in two model systems, the tgCAG-LS-TAg mice and in ovarian cancer xenografts. Despite E2's ability to accelerate disease progression in these two models, exogenous E2 did not increase the *in vivo* proliferation rate of ovarian tumours, as determined by immunostaining with the proliferation marker Ki67. In addition, E2 did not have a direct mitogenic effect on the ovarian cancer cells *in vitro*, but this may be due to most of the cell lines examined having low expression of ERα and ERβ when cultured, despite high levels of ERα expression in the tumours *in vivo.* It is possible that in these ovarian cancer models E2 may be increasing tumour growth by inhibiting apoptosis in the malignant cells. In fact, E2 has been shown to increase the expression of the pro-survival proteins bcl-2 and survivin in breast and ovarian cancer cells (Dong, Wang et al. 1999; Perillo, Sasso et al. 2000; Choi, Kang et al. 2001; Frasor, Danes et al. 2003). The amount of apoptosis in the tumours from mice treated with either E2 or placebo was not measured, but this is an

important future direction of the project and could be achieved by performing TUNEL staining. Moreover, Annexin 5 staining and flow cytometry could be used to determine the amount of apoptosis in cultured or freshly isolated mouse ascites cells treated with E2 *in vitro* or *in vivo*. We hypothesize that E2 treatment reduces the amount of apoptosis in mouse ovarian cancer cells and that a decrease in apoptosis, due to increased expression of bcl-2 and survivin, may be one mechanism through which E2 is able to increase tumour burden and ovarian cancer progression in these models of ovarian cancer.

While some potential mediators of E2 driven tumourigenesis, such as *Greb1* and *Stc2*, were identified using microarray analyses, it has yet to be determined whether E2 acts directly on the ovarian cancer cells or on the tumour microenvironment. Since many of the genes significantly up- or down- regulated by E2 (based on microarray analysis) were associated with pathways involved in angiogenesis, adhesion, differentiation and steroid hormone synthesis, it seems reasonable to speculate that the effects of E2 may be on the whole tumour environment, rather than on the ovarian cancer cells alone.

E2 has pro-inflammatory properties and can increase the release of cytokines by macrophages (Calippe, Douin-Echinard et al. 2010). However, it is doubtful that E2 has any major impact on the immune systems of the tgCAG-LS-TAg mice, because we found that exogenous E2 in immunodeficient SCID mice and tgCAG-LS-TAg mice results in a similar outcome, which is a significant acceleration of ovarian tumourigenesis. Previous studies indicate that E2 can enhance angiogenesis by healing endothelial cells and by increasing the expression of VEGF in several cell types (Arnal, Fontaine et al. 2010; Emons, Chagin et al. 2010; Ha, Joo et al. 2010; Okada, Tsutsumi et al. 2010). A better characterization of the effects of E2 on angiogenesis in the tgCAG-LS-TAg model would

be an important future direction of this project. Our microarray study indicated that VEGF receptor type 2 and endothelin receptor type A are upregulated in the tumours treated with E2, but it is not yet known if E2 stimulates proliferation and migration of endothelial cells in this model. If angiogenesis is in fact altered by E2, the tgCAG-LS-TAg model would be useful for the preclinical evaluation of anti-angiogenic and anti-estrogen therapeutics, either alone or in combination.

An important future study with this model would be to determine the effect of E2 and P4 in combination on ovarian tumour progression. Combining E2 and P4 would more accurately depict the use of oral contraceptives and/or hormone replacement therapy (HRT), since these are very rarely estrogen-only formulations. In addition, it would be beneficial to treat tgCAG-LS-TAg mice with E2 and P4 during either the early reproductive period or the late reproductive period to better represent oral contraceptive or HRT use, respectively.

A better characterization of the role of E2 in ovarian tumour initiation and progression may be achieved by using of inhibitors of estrogen signalling, such as tamoxifen, raloxifene, or letrozole. Tamoxifen may induce atrophy or cystic structures in the ovary which could complicate our interpretation of any resulting differences in ovarian cancer progression (Mourits, de Vries et al. 1999; Rehm, Solleveld et al. 2007; Harris, Chennathukuzhi et al. 2008; Mofrad, Shandiz et al. 2010), therefore it may be more useful to inhibit the actions of exogenous E2 with raloxifene. It would also be interesting to determine if decreasing the production of endogenous estrogens is sufficient to slow ovarian tumour growth in this model. This could be achieved by using the aromatase inhibitor letrozole (Jirge and Patil 2010).

An advantage of having a mouse model of ovarian cancer is the ability to study both the biology and the molecular events associated with tumour progression. We have attempted to do this by microarray analysis of ovarian tumours treated with E2 vs. placebo, and have identified several gene targets involved in cell differentiation, migration, proliferation and angiogenesis. Perhaps the most interesting candidate is gene regulated in breast cancer (Greb) 1, which is an estrogen responsive gene with a role in breast and prostate cancer growth (Rae, Johnson et al. 2005; Rae, Johnson et al. 2006). The cellular function of Greb1 is not yet known, and its expression has not previously been reported in mouse models of ovarian cancer and human ovarian tumour samples, rendering this an exciting target for further investigation using our model.

Another interesting observation made during this project was that E2 acts on the OSE cells (which express ERα and ERβ) prior to transformation, resulting in an earlier onset of tumourigenesis in the tgCAG-LS-TAg mice. Previous research in rabbits indicated that exogenous E2 results in papillary and proliferative OSE (Bai, Oliveros-Saunders et al. 2000). We demonstrated that E2 acts on the OSE cells of control mice (without ovarian tumours), creating more areas of putative preneoplastic lesions, including hyperplasia (Laviolette, Garson et al. 2010). Putative preneoplastic lesions have also been observed in human OSE after HRT use (Tok, Ertunc et al. 2006) and a large epidemiological study found that women are only at an increased risk of developing ovarian cancer while using HRT (Beral, Bull et al. 2007). Taken together, these results suggest that E2 is able to induce putative preneoplastic lesions (papillae, hyperplasia) in the OSE, which make them more susceptible for transformation, but that the OSE cell lesions and risk are only elevated while exposed to E2. Future studies designed to

compare the gene expression patterns in the preneoplastic lesions to the normal OSE are of the utmost importance for elucidating the pathways that are altered just prior to transformation and that contribute to tumour susceptibility. We anticipate that genes involved in cell polarity (Dab2) (Roland, Yang et al. 2003; Yang, D. H., Fazili et al. 2006), epithelial differentiation (E-cadherin) (MacCalman, Farookhi et al. 1994; Fleming, McQuillan et al. 2007; Ye, Xiao et al. 2010), and cell survival (survivin) (O'Neill, Deavers et al. 2005; Nabilsi, Broaddus et al. 2010) are crucial for this process. The role of E2 in ovarian cancer initiation and progression is summarized in Figure 1. We hypothesize that E2-mediated gene expression changes are responsible for an earlier onset of ovarian tumourigenesis and accelerated progression of the disease once it is initiated.

The effect of menopause on ovarian cancer progression was studied by combining the tgCAG-LS-TAg model of ovarian cancer with the VCD model of menopause. We chose the VCD model because it mimicked the hormonal (increased serum gonadotropins, decreased steroid hormones) and ovarian (increased number of epithelial inclusion cysts, remnant androgenic ovarian tissue) changes associated with menopause (Mayer, Devine et al. 2004). Importantly, VCD treatment did not harm the bursal membrane, which is crucial since tumourigenesis is initiated in the OSE of tgCAG-LS-TAg mice by intrabursally injecting AdCre. The unanticipated result that menopause prolongs survival in the tgCAG-LS-TAg mouse model of ovarian cancer, which may be due to an alteration in the histological subtype from a poorly differentiated cancer to a Sertoli tumour, underscores the importance of modelling ovarian cancer and its risk factors *in vivo* to better understand tumour biology in humans.

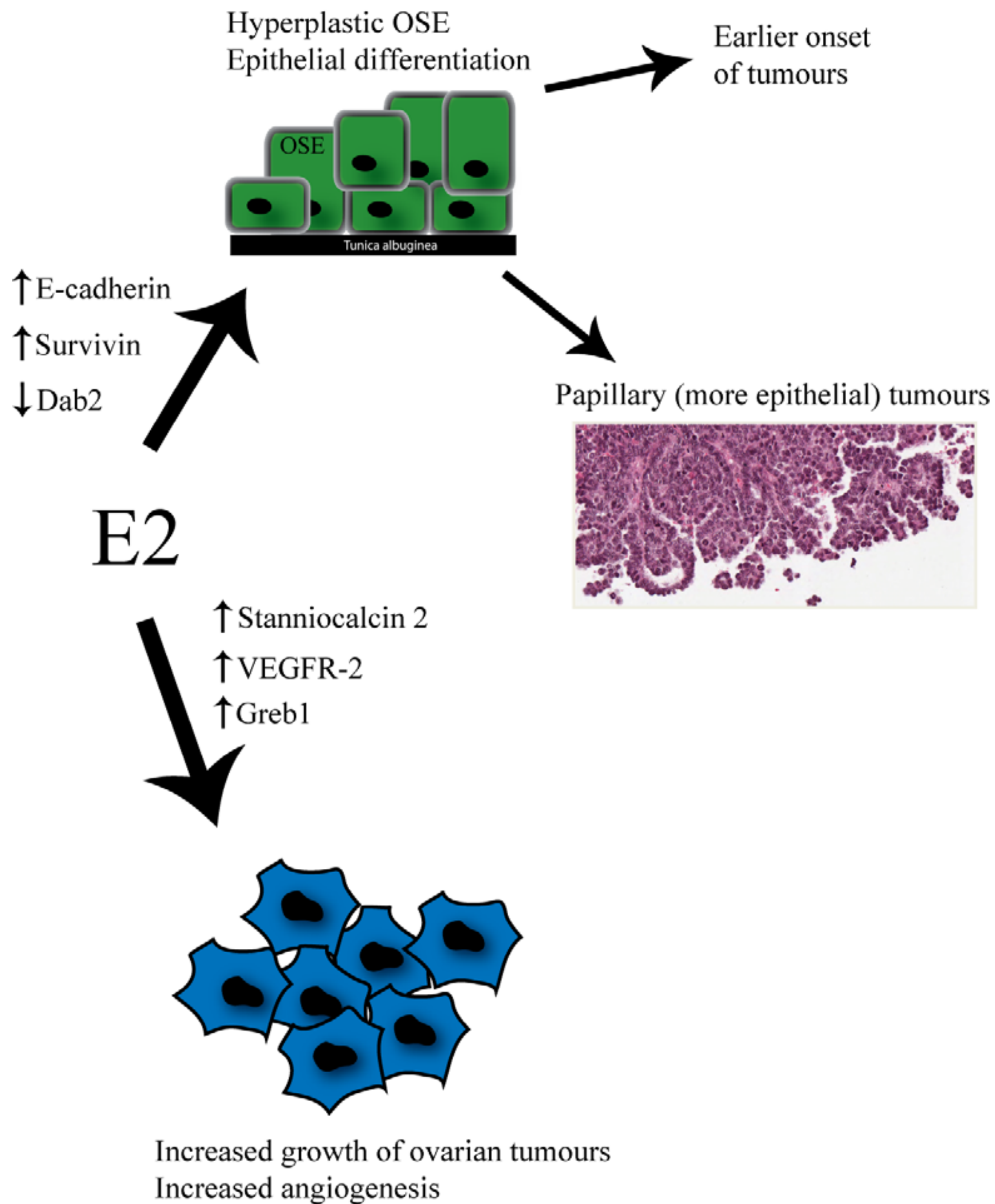

Figure 1. A model summarizing the role of E2 in the initiation and progression of ovarian cancer. It is hypothesized that E2 accelerates ovarian cancer initiation by causing epithelial differentiation and preneoplastic lesions in the OSE through the upregulation of E-cadherin and survivin and the downregulation of Dab2. The higher incidence of preneoplastic lesions in the OSE during E2 treatment makes the OSE more susceptible for malignant transformation and results in ovarian tumours which have features of epithelial differentiation (papillary histology). In addition, it is hypothesized that E2 causes increased growth and angiogenesis of established ovarian tumours through the upregulation of Stanniocalcin 2, VEGFR-2 and Greb1.

Since menopause occurs in women after many reproductive cycles, it would be interesting to utilize the VCD model to induce menopause in aged mice that have already undergone a lifetime of ovulations. This would assist us in understanding the role of ovulation in ovarian cancer etiology. Moreover, it would be interesting to treat tgCAG-LS-TAg tumourigenic mice with a combination of E2 and P4 after VCD-mediated menopause to mimic HRT use in women. Since ERα is expressed in the OSE cells and OSE epithelial inclusion cysts of VCD-treated mice, it is reasonable to speculate that the resulting tumours would respond to and be accelerated by exogenous E2.

Contributions to the field

To date, only four other transgenic models of EOC exist, one which relies on activation of the SV40 TAg oncogene throughout the reproductive epithelium, including the OSE, and three of which rely on the intrabursal administration of AdCre to knock out tumour suppressor genes or activate oncogenes. All of these models result in EOCs with either the poorly differentiated or endometrioid histological subtypes. None of these models has sufficient penetrance and latency to be used to assess the impact of disease modulators, like reproductive hormones, on EOC etiology. Moreover, these models have not been used to study the early events in disease initiation – a process that is not well understood in human ovarian cancer. We have generated a model in which cancers arise from the OSE, with sufficient latency to be useful for studying the cellular and molecular

events in disease initiation. In addition, this long latency is ideal for examining the role of potential disease modulators, such as the reproductive hormones.

It is widely accepted based on epidemiological studies that the female reproductive hormones have an impact on ovarian cancer pathophysiology. This is evidenced by the increased risk of developing ovarian cancer associated with HRT use and menopause and the decreased risk associated with oral contraceptive use and pregnancy. By utilizing a transgenic mouse to model *in situ* ovarian cancer, we found that E2 affects both ovarian cancer initiation (risk) and progression, and we have identified some genes that may be interesting therapeutic targets, such as *Greb1* and *Stc2*. The role of menopause on *in situ* ovarian cancer progression has also been reported for the first time by our group. We have indicated that transgenic mouse models of cancer can be used to elucidate the molecular and cellular events in ovarian cancer initiation and progression and suggest that this information has the potential to be translated into improved prevention and treatment strategies for women with ovarian cancer.

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