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**THE ROLE OF *Hoxa7* AND *Pax8*
IN EPITHELIAL OVARIAN CANCER DIFFERENTIATION**

By

Elizabeth Pitre

This thesis is submitted to the Faculty of Graduate and Postdoctoral Studies at the
University of Ottawa in partial fulfillment of the requirements for the degree of

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS.....	iv
ABSTRACT.....	v
LIST OF FIGURES.....	vi
LIST OF TABLES.....	viii
LIST OF ABBREVIATIONS.....	ix
 CHAPTER 1: INTRODUCTION.....	 1
1.1 Ovarian Surface Epithelium and Epithelial Ovarian Cancer.....	1
1.1.1 Ovarian Cancer Incidence Rates and Symptoms.....	1
1.1.2 Description of the Normal Ovarian Surface Epithelium.....	3
1.1.3 Invaginations and Inclusion Cysts.....	4
1.1.4 Histological Subtypes of Epithelial Ovarian Cancer.....	7
1.1.4.1 Histological and Molecular Features.....	7
1.1.4.2 Clinical Implications of Ovarian Tumour Subtype.....	10
1.1.5 Genetic and Molecular Profiles of Epithelial Ovarian Cancers Compared to Normal OSE.....	12
1.2 HOX Genes.....	13
1.2.1 Structure, Function and the Principle of Colinearity.....	13
1.2.2 HOX Gene Expression and Regulation in the Female Reproductive Tract.....	17
1.2.3 HOX Gene Expression in the Ovary.....	20
1.2.4 HOX Genes in Cancer.....	20
1.2.5 HOXA Genes in Ovarian Cancer.....	23
1.2.6 PAX8 in Ovarian Cancer.....	27
1.3 Transgenic Mouse Models of Ovarian Cancer.....	28
1.3.1 The tgMISIIR-TAG Mouse Model.....	28
1.3.2 The tgCAG-LS-TAG Mouse Model.....	30
1.4 Project Rationale.....	33
1.5 Hypothesis.....	35
1.6 Project Objectives.....	35
 CHAPTER 2: MATERIALS AND METHODS.....	 36
2.1 Tissue Culture.....	36
2.1.1 Isolation of Normal MOSE and Mouse Ovarian Cancer Cells.....	36
2.1.2 Cell Maintenance.....	39
2.1.3 Proliferation Assays.....	40
2.1.4 Assessment of Morphology.....	40
2.1.5 Treatment of Cells with EGF.....	41
2.1.6 Ascites Cell Growth in Suspension.....	42

2.2	RNA Analysis.....	43
2.2.1	Extraction and Quantification of RNA.....	43
2.2.2	Reverse Transcription-PCR.....	44
2.2.3	Quantitative-PCR.....	45
2.3	Lentiviral Transduction.....	47
2.3.1	Lentiviral Constructs.....	47
2.3.2	Lentivirus Production.....	49
2.3.3	Lentiviral Transduction of Cell Lines.....	51
2.3.4	Confirmation of Transgene Expression.....	51
2.4	Assessment of Lentivirally-Transduced Cells.....	52
2.4.1	Proliferation Assays.....	52
2.4.2	Assessment of Morphology.....	53
2.4.3	Protein Analysis.....	53
2.4.4	Colony Formation Assays.....	55
2.5	Immunohistochemistry.....	56
2.6	Statistical Analyses.....	58
CHAPTER 3: RESULTS.....		61
3.1	Cell line proliferation rates and morphology.....	61
3.2	Endogenous expression levels of <i>Hoxa</i> and <i>Pax8</i> genes.....	64
3.3	Effects of <i>Hoxa7</i> overexpression.....	69
3.4	<i>Hoxa7</i> expression in tumours and ascites.....	85
3.5	Effect of <i>in vitro</i> culture conditions on <i>Hoxa7</i> expression.....	93
3.6	<i>Pax8</i> expression in tgMISIIR-TAg and tgCAG-LS-TAg tumours.....	96
CHAPTER 4: DISCUSSION.....		104
REFERENCES.....		120

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ABSTRACT

Epithelial ovarian cancer (EOC) is divided into several histological subtypes characterized by Müllerian-like differentiation and the expression of genes not expressed in normal ovarian surface epithelium. Many of these genes are implicated in female reproductive tract development, including *Hoxa7* and *Pax8*, and it has been proposed that these genes are involved in the differentiation and progression of EOC. The effects of *Hoxa7* overexpression (cellular proliferation, morphology, expression of E-cadherin and vimentin) were investigated in cell lines derived from our transgenic mouse models of ovarian cancer. However, *Hoxa7* overexpression did not significantly affect any of these parameters. Immunohistochemical analysis revealed that Pax8 often appears to be localized to regions of papillary differentiation in tumours from transgenic tgMISIIR-TAg mice. These results suggest that while *Hoxa7* alone does not drive EOC differentiation *in vitro*, expression of *Pax8* may contribute to the differentiated epithelial features of ovarian cancer.

LIST OF FIGURES

Figure 1:	Invaginations and inclusion cysts in the ovary.....	6
Figure 2:	Organization of mammalian <i>HOX</i> genes.....	15
Figure 3:	The tgMISIIR-TAG transgene construct.....	29
Figure 4:	Induction of TAG expression in tgCAG-LS-TAG mice.....	31
Figure 5:	<i>Hoxa7</i> and <i>18S</i> dissociation curves and confirmation of band sizes.....	48
Figure 6:	<i>Hoxa7</i> lentivirus vector map.....	50
Figure 7:	Photomicrographic images of normal MOSE and mouse ovarian cancer cell lines.....	62
Figure 8:	Proliferation rates of normal MOSE and mouse ovarian cancer cell lines.....	63
Figure 9:	Endogenous <i>Hoxa7</i> , <i>Hoxa9</i> , <i>Hoxa10</i> , and <i>Hoxa11</i> mRNA levels in normal MOSE and mouse ovarian cancer cell lines by Q-PCR.....	65
Figure 10:	Endogenous <i>Pax8</i> expression in normal MOSE and mouse ovarian cancer cell lines.....	68
Figure 11:	GFP expression detected by flow cytometry in mixed populations of WPI-LV and <i>Hoxa7</i> -LV-transduced cells.....	70
Figure 12:	GFP expression in <i>Hoxa7</i> -LV and WPI-LV transduced, sorted cells.....	75
Figure 13:	Overexpression of <i>Hoxa7</i> in <i>Hoxa7</i> -LV-transduced cells.....	76
Figure 14:	<i>Hoxa7</i> overexpression does not affect growth rate in normal MOSE or mouse ovarian cancer cells over 96 hours.....	78
Figure 15:	Photomicrographic images of lentivirally-transduced MOSE and mouse ovarian cancer cell lines.....	79
Figure 16:	E-cadherin and vimentin protein expression in normal MOSE and mouse ovarian cancer cell lines.....	81

Figure 17:	Overexpression of <i>Hoxa7</i> does not affect E-cadherin or vimentin protein levels in normal MOSE or mouse ovarian cancer cell lines.....	82
Figure 18:	Overexpression of <i>Hoxa7</i> does not enhance colony formation in normal MOSE.....	84
Figure 19:	Cultured ascites cell lines express more <i>Hoxa7</i> than primary tumours from the same mouse model of EOC.....	86
Figure 20:	<i>TA</i> normalization does not change the pattern of <i>Hoxa7</i> expression in cell lines compared to primary tumours.....	87
Figure 21:	<i>Hoxa9</i> expression in cultured cell lines and primary tumours from mouse models of EOC.....	88
Figure 22:	<i>Hoxa10</i> expression in cultured cell lines and primary tumours from mouse models of EOC.....	90
Figure 23:	Uncultured SCID mouse ascites express less <i>Hoxa7</i> than their paired SCID mouse tumours from derived from xenograft studies.....	91
Figure 24:	<i>TA</i> normalization does not change the pattern of <i>Hoxa7</i> expression SCID mouse ascites compared to their paired SCID mouse tumours derived from xenograft studies.....	92
Figure 25:	EGF has no effect on <i>Hoxa7</i> expression <i>in vitro</i> in normal MOSE and mouse ovarian cancer cell lines.....	94
Figure 26:	EGF affects the morphology and E-cadherin expression of normal MOSE and mouse ovarian cancer cells <i>in vitro</i>	95
Figure 27:	Suspension culture does not affect <i>Hoxa7</i> expression in mouse ascites cell lines.....	97
Figure 28:	<i>Pax8</i> is expressed in the mouse uterus and oviduct, but not in the OSE...	98
Figure 29:	Ovarian tumours from tgCAG-LS-TAg mice express little <i>Pax8</i>	100
Figure 30:	<i>Pax8</i> expression in tumours from tgMISIIR-TAg mice.....	102

LIST OF TABLES

Table 1:	MOSE and mouse ovarian cancer cell lines that were used in this study	37
Table 2:	Percentage of GFP+ cells in a mixed population of transduced and untransduced cells.....	73
Table 3:	Time required to achieve a two-fold difference in GFP+ <i>Hoxa7</i> cells compared to untransduced or GFP+ WPI control cells.....	74

LIST OF ABBREVIATIONS

α -MEM	alpha minimum essential medium
AdCre	adenovirus expressing Cre recombinase
AdGFP	adenovirus expressing GFP
AML	acute myeloid leukemia
bp	base pairs
CA 125	cancer antigen 125
CAG	cytomegalovirus early enhancer/chicken β -actin promoter
cDNA	complementary DNA
cm	centimetre(s)
DAB	diaminobenzidine
DBS	donor bovine serum
ddH ₂ O	double distilled H ₂ O
DES	diethylstilbestrol
D-MEM	Dulbecco's minimum essential medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
E2	17 β -estradiol
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EMA	epithelial membrane antigen
EMT	epithelial to mesenchymal transition
EOC	epithelial ovarian cancer
EpCAM	epithelial cell adhesion molecule
Erk	extracellular signal-regulated kinase
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
FSH	follicle stimulating hormone
GFP	green fluorescent protein
H ₂ O	water
HCl	hydrochloric acid
HRP	horseradish peroxidase

IgG	Immunoglobulin G
IHC	immunohistochemistry
ILK	integrin-linked kinase
IP	intraperitoneal
IRES	internal ribosome entry site
LH	luteinizing hormone
LMP	low melting point
LV	lentivirus
µg	microgram(s)
µl	microlitre(s)
µm	micrometre(s)
µM	micromolar
M	molar
MASC2	mouse ascites control 2, an ovarian cancer cell line
MASE2	mouse ascites estrogen 2, an ovarian cancer cell line
MASP1	mouse ascites progesterone 1, an ovarian cancer cell line
mg	milligram(s)
MISIIR	Müllerian-inhibiting substance type II receptor
MISIIR-TAg	cell line derived from the ovarian tumour of a tgMISIIR-TAg mouse
ml	millilitre(s)
mm	millimetre(s)
mM	millimolar
MOPS	3-(N-morpholino)propanesulfonic acid
MOSE	mouse ovarian surface epithelium
MOSE-RM	ovarian surface epithelial cell line from an Immortomouse that has been transformed by retroviral infection with <i>K-ras</i> ^{G12D} and <i>c-myc</i>
NaCl	sodium chloride
ng	nanogram(s)
OSE	ovarian surface epithelium
OGP	oviduct-specific glycoprotein
P4	progesterone
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Q-PCR	quantitative polymerase chain reaction
RNA	ribonucleic acid

rpm	rotations per minute
RT	reverse transcriptase
RT-PCR	reverse transcription polymerase chain reaction
SCID	severe combined immunodeficiency
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
S-PBS	Stockholm phosphate buffered saline
TAg	large and small Simian virus 40 T antigens
TBST	tris-buffered saline Tween-20
tgMISIIR-TAg	transgenic MISIIR-TAg mouse model of ovarian cancer
tgCAG-LS-TAg	transgenic CAG-LS-TAg mouse model of ovarian cancer
U	units
V	volts
WT1	Wilms tumor 1 protein

CHAPTER 1: INTRODUCTION

1.1 Ovarian Surface Epithelium and Epithelial Ovarian Cancer

1.1.1 Ovarian Cancer Incidence Rates, Symptoms and Treatment

Ovarian cancer is an aggressive malignancy that has the highest mortality rate of all gynecological cancers. Approximately 90% of all ovarian cancers are epithelial cancers and are thought to develop from the ovarian surface epithelium (OSE), a simple squamous, cuboidal, or pseudostratified columnar mesothelium that lines the surface of the ovary (Auersperg *et al.*, 2001). Several risk factors (family history, menopause, nulliparity, the use of hormone replacement therapy) and protective factors (increasing parity and the use of oral contraceptives) have been identified for ovarian cancer from epidemiological studies, though the precise etiology of epithelial ovarian cancer (EOC) remains unclear (Sueblinvong and Carney, 2009). Although germline mutations in the *BRCA1* or *BRCA2* tumour suppressor genes are well established as strong predictors of developing ovarian cancer, only 10% of all ovarian cancers can be attributed to inherited mutations, and the remainder of cases arise spontaneously (Palma *et al.*, 2006).

A Canadian woman's lifetime risk of developing ovarian cancer is about 1 in 71 women, and the five-year survival rate for women diagnosed with this disease is 40% (Canadian Cancer Society Statistics 2009). This poor long term survival rate is primarily because the majority of patients present with advanced-stage disease (Stage III/IV), when the cancer has already metastasized to other sites in the peritoneal cavity and beyond. Late-stage presentation is so common because there are no screening methods for early

detection of ovarian cancer. The most widely used detector of ovarian cancer is the serum level of cancer antigen 125 (CA 125), however these levels are only elevated in around half of cases of early-stage disease and in 80% of women with advanced disease (Nossov *et al.*, 2008). In addition, symptoms of ovarian cancer are vague or completely absent. When symptoms do present, they usually mimic those of gastrointestinal and bladder disorders, including persistent bloating, constipation, urinary urgency and pelvic pain. Such symptoms can easily be dismissed by women and their doctors, further contributing to late-stage ovarian cancer detection.

Treatment regimens vary according to the age of the patient, and the stage and grade of the cancer, but standard options include surgical resection of as much tumour tissue as possible, followed by intravenous or intraperitoneal (IP) administration of taxane and platinum-based chemotherapeutic drugs such as paclitaxel, cisplatin and carboplatin. Primary surgery often also includes removal of the uterus, fallopian tubes, contralateral ovary, and omentum. When this initial cytoreductive surgery is optimally performed in women with Stage I or Stage II ovarian cancer with postoperative adjuvant chemotherapy, the response rate is very good and overall survival ranges from 80-95% (Cannistra, 2004). However, approximately 70% of patients with late-stage ovarian cancer will relapse, and often the recurrent disease is chemo-resistant (Högberg *et al.*, 2001). This makes successful, long-term treatment of EOC very challenging and increases the need for better therapies. Unfortunately, the initiating and early molecular events in ovarian carcinogenesis are poorly understood, which creates a barrier to developing early detection strategies and appropriate, ovarian tumour-targeted treatments.

1.1.2 Description of the Normal Ovarian Surface Epithelium

The OSE is a simple squamous, cuboidal, or pseudostratified columnar mesothelium that lines the surface of the ovary (Auersperg *et al.*, 2001). It is embryonically derived from the coelomic epithelium, which is also the source of the embryonic Müllerian ducts from which the structures of the female reproductive tract (the fallopian tubes in humans and oviducts in mice, uterus, cervix, and vagina) arise (Hashimoto, 2003; Guioli *et al.*, 2007). OSE in the adult has an uncommitted phenotype both *in vivo* and *in vitro*. Cultured OSE are capable of acquiring a cobblestone-like appearance that is characteristic of epithelial cells, and an elongated phenotype with projections, similar to fibroblasts (Auersperg *et al.*, 1994). *In vivo*, OSE can normally be either squamous or cuboidal, and this phenotypic plasticity is mirrored by a functional plasticity. In response to ovulation and the rupturing of the follicle and ovarian surface, the OSE must be able to rapidly proliferate to close the ovulatory wound. Indeed, OSE directly adjacent to the site of ovulation have been shown to be cuboidal in shape and more highly proliferative than squamous OSE that were not associated with these sites (Tan and Fleming, 2004). However, the factors stimulating OSE proliferation are not completely understood; while murine OSE respond to follicle stimulating hormone (FSH) and luteinizing hormone (LH) stimulation *in vivo* (Davies *et al.*, 1999), their proliferation does not increase in the presence of estrogen, a known mitogen (Laviolette *et al.*, 2010).

The OSE can be histologically distinguished from other mesothelia by the presence of cilia (Blaustein and Lee, 1979), but there are no known OSE-specific cell surface markers that enable sorting for OSE from a mixed population of mesothelial cells

by fluorescence-activated cell sorting (FACS), for example. However, since the OSE is so delicately attached to its basement membrane, it is possible to remove it from the ovary by gentle surgical scraping or through enzymatic means (Kruk *et al.*, 1990). Both mouse and human OSE express cytokeratins, which are typical epithelial cell markers (Auersperg *et al.*, 1984; Quirk *et al.*, 1997), so OSE can be identified in culture at the exclusion of all other ovarian cell types. Along with cytokeratins, human OSE constitutively express N-cadherin, which is an adhesion molecule that forms cell-cell junctions in mesodermally-derived epithelia (Wong *et al.*, 1999). E-cadherin, which is the cadherin family member usually expressed in epithelia, is only conditionally expressed in human OSE (Maines-Bandiera and Auersperg, 1997; Wong *et al.*, 1999) and is expressed at low levels in murine OSE (Fleming *et al.*, 2007). Although the OSE are epithelial cells, both mouse and human OSE also express vimentin, further evidence of an uncommitted state of differentiation (Jiang *et al.*, 2003; Davies *et al.*, 2003). The OSE also expresses an array of receptors, including those for LH and FSH (Davies *et al.*, 1999; Lu *et al.*, 2000; Syed *et al.*, 2001), estrogen and progesterone (Brandenberger *et al.*, 1998; Lau *et al.*, 1999), and epidermal growth factor (EGF) (Doraiswamy *et al.*, 2000). KIT, a receptor tyrosine kinase, is not expressed in normal human OSE, but the expression of KIT Ligand is detectable in normal human and rat OSE (Tonary *et al.*, 2000; Ismail *et al.*, 1999).

1.1.3 Epithelial Invaginations and Inclusion Cysts

Though the OSE generally lies on the surface of the ovary, it has the capacity to invade the ovarian cortex and form clefts referred to as invaginations. If the OSE cells of

these invaginations pinch off from the surface entirely, they can form OSE-lined inclusion cysts (**Figure 1**). It has also been proposed that inclusion cysts can be formed when the OSE close to an ovulating follicle becomes trapped within the stroma after the follicle and ovarian surface rupture (Murdoch, 1994). The OSE of invaginations and inclusion cysts differs from surface OSE regarding its morphology and expression profiles. OSE cells within inclusion cysts are more often columnar than squamous (Maines-Bandiera and Auersperg, 1997). They also begin to express proteins not commonly seen in normal OSE, including E-cadherin (Maines-Bandiera and Auersperg, 1997; Sundfeldt *et al.*, 1997), KIT (Tonary *et al.*, 2000), HOXA7 (Naora *et al.*, 2001a) and several fallopian tube/oviduct markers, including PAX8 (Bowen *et al.*, 2007), oviduct-specific glycoprotein (OGP) (Woo *et al.*, 2004), epithelial membrane antigen (EMA), epithelial cell adhesion molecule (EpCAM), and mesothelin (Drapkin, 2004). These altered cell morphologies and protein expression profiles are more characteristic of a committed, differentiated epithelium than of normal surface OSE, indicating Müllerian-like differentiation in particular.

While invaginations and inclusion cysts are benign and can be found in grossly normal ovaries, they are considered to be putative pre-neoplastic lesions (Auersperg *et al.*, 2001). There are epidemiological links between invaginations or inclusion cysts and the risk factors for EOC, as these lesions are more commonly seen in the ovaries of post-menopausal women (Tok *et al.*, 2006) and in women with a family history of ovarian cancer, including *BRCA1* mutation carriers (Werness *et al.*, 1999; Salazar *et al.*, 1996). Furthermore, not only are inclusion cysts more prevalent in the contralateral ovaries of

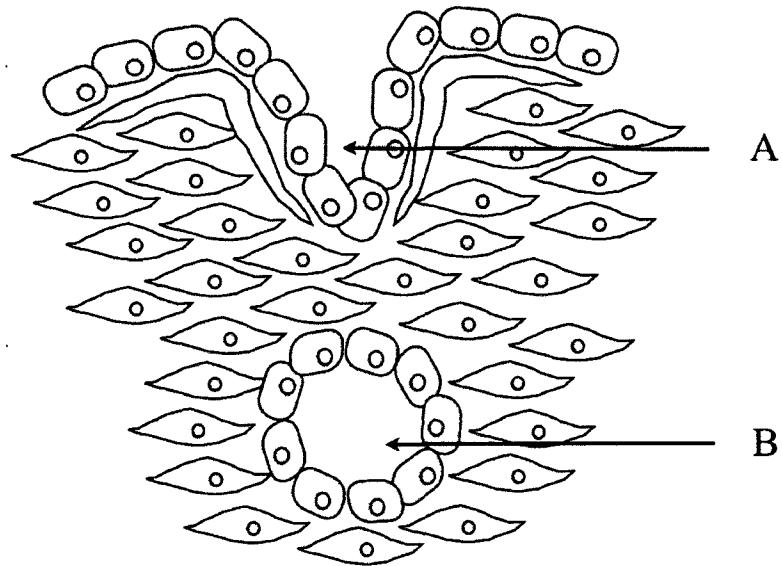


Figure 1: Invaginations and inclusion cysts in the ovary. (A) An invagination of OSE cells into the stroma of the ovary. (B) An inclusion cyst within the stroma, made up of OSE cells which have the propensity to undergo metaplasia, dysplasia, and potentially transformation.

women with serous ovarian tumours than in the ovaries of women without ovarian cancer (Mittal *et al.*, 1993), they can also sometimes be found next to the serous tumour itself (Bell and Scully, 1994). Molecular similarities between inclusion cysts and established EOC have also been demonstrated; all the proteins listed above that are aberrantly expressed in cortical inclusion cysts are also expressed in ovarian carcinomas. The Müllerian-like differentiation seen in inclusion cysts is also commonly seen in EOC, which will be discussed further in section 1.1.4. There is also some evidence that the OSE within inclusion cysts is abnormal and is capable of initiating tumourigenesis. Namely, assessments of ovarian inclusion cysts have revealed genomic instability not found as commonly in nearby normal OSE that is similar to serous ovarian carcinomas (Körner *et al.*, 2005), and there have been some reports of microscopic cancerous lesions that occupy the site of ovarian inclusion cysts (Scully, 1995).

1.1.4 Histological Subtypes of Epithelial Ovarian Cancer

1.1.4.1 Histological and Molecular Features

The progression of EOC is quite unique; ovarian tumours display a much more differentiated phenotype than that of the OSE, their presumed tissue of origin. While OSE have characteristics of both epithelial and stromal cells and often exhibit a rather unremarkable phenotype, epithelial ovarian tumours can be incredibly structured and organized. Specifically, epithelial ovarian tumours often fit at least one of several defined histological subtypes, the most common of which are the serous, endometrioid, and mucinous subtypes (Auersperg *et al.*, 2001). The vast majority (80-85%) of all ovarian

carcinomas are classified as serous, 10% of tumours are endometrioid, and the rest are either mucinous or one of the other more rare subtypes (Seidman *et al.*, 2004). These classifications are given based on the morphological resemblance of the tumours to structures of the normal, Müllerian duct-derived female reproductive tract; serous tumours have a papillary phenotype much like the fallopian tube, endometrioid carcinomas have glandular structures reminiscent of uterine endometrial glands, and mucinous carcinomas resemble cervical epithelium. The definitive cause of this histotype-specific tumour differentiation remains unknown.

While the majority of ovarian carcinomas can be classified as serous, there is quite a bit of morphological and genetic heterogeneity within this group. This makes grading of serous tumours somewhat difficult, as there are multiple classification systems that all have slightly different criteria for this tumour characteristic. In general, tumours are graded based on architecture, histology, and mitotic index; low-grade tumours are defined as well differentiated lesions with low proliferative activity, whereas high-grade tumours are poorly differentiated, aggressive tumours with a high mitotic index. The distinction between low-grade and high-grade serous tumours is critical not only because they are morphologically dissimilar, but also because there is increasing evidence that these tumours develop from distinct molecular pathways. It has been proposed that low-grade serous carcinomas have more in common with the non-serous ovarian cancer subtypes than with high-grade serous tumours, as the former group develop in a stepwise fashion from borderline precursors, but the latter may arise *de novo* from the OSE or inclusion cysts (Shih and Kurman, 2004). The molecular profiles of these different

tumour subtypes are quite discrete, which supports this hypothesis. Mutations in the proto-oncogenes *BRAF* or *KRAS* have been reported in almost 70% of low-grade serous carcinomas, but not in any high-grade serous carcinomas (Singer *et al.*, 2003). In contrast, *TP53* mutations are rare in low-grade serous tumours but are detected in more than half of poorly differentiated, high-grade serous tumours (Milner *et al.*, 1993; Singer *et al.*, 2005). The expression of Wilms tumor 1 protein (WT1) has also been widely used as an immunohistochemical marker of high-grade serous ovarian carcinomas, as it can be detected in up to 90% of these tumours (Köbel *et al.*, 2009).

The serous subtype of ovarian cancer is not the only histotype that is associated with a specific set of molecular mutations and alterations. Somatic mutations in the β -catenin gene *CTNNB1* and the tumour suppressor gene *PTEN* are predominant within the endometrioid histotype (Palacios and Gamallo, 1998; Obata *et al.*, 1998). The *CTNNB1* mutations, which are present in approximately 30% of endometrioid tumours, are localized to the sequence associated with ubiquitin-mediated degradation by the proteasome, leading to the accumulation of β -catenin in the nucleus (Gatcliffe *et al.*, 2008). This nuclear as opposed to membranous localization of accumulated β -catenin is also specific to the endometrioid ovarian subtype (Gilks, 2010). Furthermore, it has recently been investigated whether the dual pathway hypothesis that has been suggested for low and high-grade serous tumours is also applicable for low and high-grade endometrioid tumours; in a study of 29 tumour samples, it was reported that *CTNNB1* and *PTEN* mutations were preferentially found in low-grade carcinomas (Geyer *et al.*, 2009).

Mucinous carcinomas only make up 3-4% of all ovarian carcinomas, and when they occur they are usually limited to the ovary (Gilks, 2010). Mutations in codons 12 and 13 of the *KRAS* gene can be found in this subtype, which is also true of the low-grade serous subtype. However, unlike low-grade serous tumours, *KRAS* mutations are the most commonly detected mutation; *KRAS* mutations at these loci were present in 85% of mucinous carcinoma samples (Cuatrecasas *et al.*, 1997), compared to 35% of low-grade serous carcinomas (Singer *et al.*, 2003). Furthermore, mucinous ovarian carcinomas can be molecularly distinguished from low-grade serous carcinomas by the presence of *HER2* amplification in approximately 20% of cases, an event that is rare in the other ovarian cancer subtypes (McAlpine *et al.*, 2009).

1.1.4.2 Clinical Implications of Ovarian Tumour Subtype

Ovarian tumour histotype, stage and grade are critical determinants of patient response to treatment and overall survival. The Gynecologic Oncology Group published a study indicating that histotype is an independent predictor of prognosis, specifically that the mucinous subtype is associated with poor progression-free survival (Winter *et al.*, 2007). This is perhaps not surprising, given that mucinous tumours are well known to be resistant to standard chemotherapies (Hess *et al.*, 2004). The recent discovery of *HER2* amplification in mucinous ovarian carcinomas lead to the promising preliminary study of trastuzumab, a monoclonal antibody to *HER2*, in patients with this tumour type; this showed that one patient (of three) with recurrent disease had a dramatic response to trastuzumab (McAlpine *et al.*, 2009).

However, it has also been suggested that the recognition of so many distinct histotypes is not as clinically relevant as categorizing carcinomas as either Type I or Type II tumours (Shih and Kurman, 2004). This classification primarily differentiates tumours based on grade; Type I tumours are generally low-grade, including low-grade serous and endometrioid carcinomas, as well as most mucinous tumours. In addition, there are morphologically recognizable benign or borderline precursor lesions associated with these tumours. Type II tumours are generally high-grade serous and endometrioid tumours, with unknown precursor lesions. Given that Type I tumours arise from borderline or benign tumours which generally remain slow growing and confined to the ovary, they are easier to detect at an earlier stage and sometimes easier to treat than Type II tumours (Cho and Shih, 2009). This means that women with Type I tumours generally exhibit better clinical outcomes than those with Type II tumours. Comparisons in survival rate between women with different histotypes of ovarian cancer often show that survival relies more on the grade of the tumour than on the histotype itself. For example, patients with low-grade serous carcinomas (about 10% of all serous tumours) tend to present at a younger age and have a better overall survival rate than those with high-grade tumours of any subtype (Gershenson *et al.*, 2006), and endometrioid ovarian adenocarcinomas are usually low-stage and low-grade, so patients with this subtype of ovarian tumour generally live longer than those with serous tumours (Gilks, 2010).

1.1.5 Genetic and Molecular Profiles of Epithelial Ovarian Cancers Compared to Normal OSE

Along with the acquisition of a differentiated tumour phenotype that resembles structures in the female reproductive tract, EOC is also characterized by the expression of genes and proteins not normally expressed in the OSE. For example, EOCs of all subtypes express E-cadherin (Fujimoto *et al.*, 1997) and HOXA7 (Cheng *et al.*, 2005), and certain subtypes express HOXA9, HOXA10, HOXA11 (Cheng *et al.*, 2005), and PAX8 (Bowen *et al.*, 2007). In fact, the gene expression profiles of each ovarian cancer subtype are more similar to epithelium from the normal structures that they morphologically resemble than to normal OSE; gene expression in serous carcinomas correlates with fallopian tube gene expression but not with other EOC subtypes or normal OSE, for example (Marquez *et al.*, 2005). As was mentioned previously, E-cadherin, HOXA7, and PAX8 can also be found in inclusion cysts. Therefore, since aberrant positioning of OSE cells within the ovary as inclusion cysts as opposed to on the surface is associated with the activation of genes that are expressed in cancer but not in normal OSE, inclusion cysts have been identified as likely intermediate structures in the initiation of EOC. However, it is unclear whether this aberrant gene expression in inclusion cysts drives cellular transformation and the differentiation of EOC or whether it is a consequence of this transformation and differentiation.

1.2 *HOX* Genes

1.2.1 Structure, Function and the Principle of Colinearity

HOX genes are members of a highly conserved gene family that confer positional identity in the developing embryo. *HOX* genes and their function were first identified in *Drosophila melanogaster*, as mutations in these genes caused homeotic transformations, or the transformation of one body segment to another. For example, a mutation in the *Antp* gene resulted in the development of a leg at the position where there should normally be an antenna (Lewis, 1956). *HOX* genes are relatively small genes, containing only one intron of varying length and two exons. They are defined by a 183 base pair (bp) DNA sequence residing in the second exon called the homeobox. The homeobox encodes the highly conserved, 61 amino acid homeodomain, which is a helix-turn-helix DNA binding domain (Lappin *et al.*, 2006).

The *HOX* genes in *Drosophila* exist as clusters within two gene complexes. The *bithorax* complex contains three *HOX* genes (*Ubx*, *Abd-A*, *Abd-B*) and the *Antennapedia* complex contains five *HOX* genes (*Lab*, *Pb*, *Dfd*, *Scr*, *Antp*). The first mammalian genes showing homology to the *Drosophila* homeobox were characterized in the mouse by using *Drosophila* homeobox probes against murine DNA (McGinnis *et al.*, 1984). Since these initial studies, *HOX* genes have been identified in all animals, plants, and fungi, evidencing their early evolutionary origins (Lappin *et al.*, 2006). However, different organisms have different numbers of *HOX* genes and complexes; this indicates that several duplication events of single genes or whole clusters have occurred in various species over the course of evolution, and all *HOX* genes have been proposed to arise from

a single, hypothetical *ProtoHox* gene (Garcia-Fernandez, 2005). There are 39 *HOX* genes in mammals which are organized into four clusters, named A to D. Each cluster is positioned on a different chromosome (7, 17, 12 and 2, respectively for human and 6, 11, 15 and 2 respectively for mouse), and within each cluster are a number of adjacently positioned *HOX* genes that belong to one of 13 paralog groups based on sequence identity and relative position on the chromosome (Scott, 1992) (**Figure 2**).

A fascinating feature of *HOX* genes in vertebrates is that they exhibit both spatial and temporal colinearity. Spatial colinearity was established first in *Drosophila* when it was shown that the *HOX* genes were clustered on the chromosome in a colinear fashion to their position of expression along the thorax and abdomen; the 3' genes in the cluster were expressed at the most anterior body position and the 5' genes are expressed in the most posterior body compartments (Lewis, 1978). Years later, temporal colinearity was also demonstrated in mice and has since been shown to occur in all vertebrates, meaning that 3' *HOX* genes are also expressed earlier in the embryo than the 5' genes of the cluster (Dollé and Duboule, 1989). Therefore, expression of the 3' *HOX* genes is responsible for patterning anterior body segments and expression of 5' *HOX* genes patterns posterior body segments. This also means that there is an overlap of transcript expression and an increasing amount of *HOX* products towards the posterior end of the body, and it is not possible to detect any given *HOX* transcript until the *HOX* genes 3' to that position have been expressed. Therefore, positional identity is necessarily defined by the most posterior *HOX* gene expressed at a given body segment, an idea that has been termed “posterior prevalence” (Duboule, 1991). It follows, then, that if expression of the most posterior

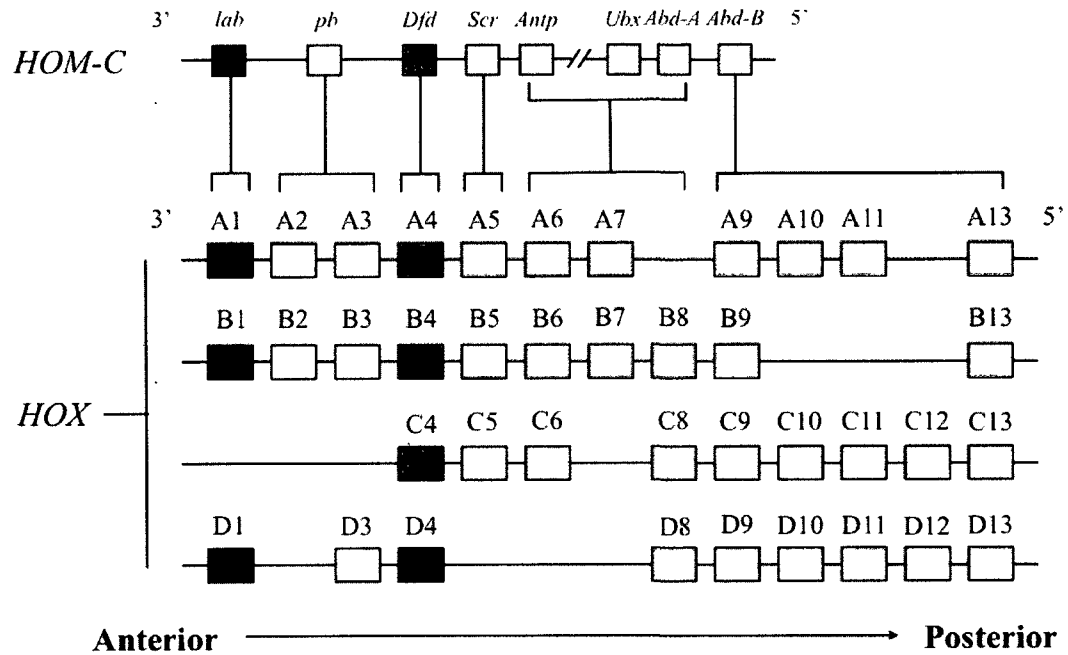


Figure 2: Organization of *HOX* genes. There are 39 mammalian *HOX* genes in four clusters, each on a separate chromosome. Each *HOX* gene belongs to one of 13 paralog groups which are highly homologous to the *Drosophila HOM-C* genes. *HOX* genes display spatial and temporal colinearity; the 3' genes are expressed in anterior body segments of the embryo and earlier, while the 5' genes are expressed in posterior body segments and later. Adapted from Lappin *et al.*, 2006.

HOX gene at a given position is suppressed, the default identity assumed at that location will be that of the one defined by the next 3' *HOX* gene. Indeed, this has been observed in numerous *HOX* gene mutants. For example, null mutations in *Hoxc8*, a gene involved in vertebral differentiation, result in the homeotic transformation of the first lumbar vertebra for a more anterior thoracic vertebra in the mouse (Le Mouellic *et al.*, 1992). Conversely, overexpression of the posterior homeobox gene *XIHbox 6* in the anterior compartment of *Xenopus* embryos is sufficient to drive the formation of tail structures where the head would normally develop (Cho *et al.*, 1991). There have been a few regulatory mechanisms suggested which would allow for such spatial and temporal colinearity. The first possibility is that the transcription of early genes leads to chromatin remodeling and increasing transcriptional accessibility to successive *HOX* genes. Another possibility is that there are global enhancer sequences which exist outside the *HOX* cluster that differentially regulate gene expression as a result of varying distance from each gene, promoter competition or affinity (Kmita and Duboule, 2003).

As all *HOX* gene products contain the DNA binding homeodomain, their main function is to act as transcription factors that regulate gene expression during embryonic development and often in adult tissues. The target gene binding specificity is provided not by the *HOX* gene sequence, since the homeobox is highly conserved, but rather through interactions with several cofactors in large protein complexes. The main outcome of these actions is embryonic axial patterning and cell fate determination. *HOX* genes are first expressed in the embryo at the time of gastrulation, when the three germ layers are formed and the body axis is established (Gruss and Kessel, 1991). Demonstrating the

principal of spatial and temporal colinearity, the *HOX1* paralogs are expressed initially at the anterior end of the primitive streak, and the more 5' paralogs are expressed as the axis is elongated posteriorly. The role of *HOX* genes in body patterning and cell fate determination throughout embryogenesis has been well established in many different organ systems and processes, including limb development (Zakany and Duboule, 2007), hematopoiesis (Sauvageau *et al.*, 1994), and development of the female reproductive tract (Kobayashi and Behringer, 2003).

1.2.2 *HOX* Gene Expression and Regulation in the Female Reproductive Tract

The development of the female reproductive tract begins shortly after gastrulation with the regression of the Wolffian ducts (the presumptive male reproductive tract) and differentiation of the Müllerian ducts, which contain epithelial cells surrounded by mesenchyme. Several homeodomain-containing genes are involved in the proper development of the fallopian tubes, uterus, cervix and vagina, including the *Abd-B*-like *HOXA* genes, *HOXA9*, *HOXA10*, *HOXA11*, and *HOXA13*. Expression of these genes has been shown in the Müllerian ducts and reproductive tracts of female mouse embryos, neonates and adults, and a conserved *HOX/Hox* axis has been established in the adult female reproductive tract of both human and mouse ("*HOX*" in humans, "*Hox*" in mice; Taylor *et al.*, 1997). In the mouse, the *Hoxa* genes are uniformly expressed in the undifferentiated Müllerian ducts, but their expression was maximal and spatially restricted between birth and two weeks of age, when the differentiation of the Müllerian

ducts was at its peak. At this time, *Hoxa9* was predominantly expressed in the oviducts, *Hoxa10* was expressed in the uterine stroma and epithelium, *Hoxa11* was expressed in the uterus and cervix, and *Hoxa13* was strongly expressed in the vagina. Furthermore, this expression pattern was maintained in the adult mouse and was paralleled in the adult human. It is also required for proper reproductive tract development and positional identity; *Hoxa10* and *Hoxa11* mutant mice are infertile as a result of the inability of the uterus to develop properly and allow for zygote implantation (Satokata *et al.*, 1995; Hsieh-Li *et al.*, 1995). This uterine-factor infertility in *Hoxa10* knockout mice is perhaps not surprising since the anterior uterine segment of these mice undergoes homeotic transformation to oviduct-like structures (Benson *et al.*, 1996).

The factors regulating *HOX* gene expression and their influence on differentiation and patterning are complex and poorly understood. In addition to spatial and temporal regulation of *HOX* gene expression, the binding of various cofactors like Meis and Pbx proteins are integral to proper *HOX* functioning (Moens and Selleri, 2006), and recently, endocrine regulation of *HOX* genes has also been demonstrated. The epithelium in the adult female reproductive tract undergoes extensive turnover throughout the menstrual cycle, so expression of the *HOX* genes into adulthood is thought to be necessary for these cells that have to repeatedly differentiate, and this may be facilitated by estrogen and progesterone. Endocrine regulation of *HOX* genes has been studied the most in the uterus. *HOXA10* and *HOXA11* transcript levels are the highest during the mid-secretory stage, at which point the endometrium has differentiated in preparation for implantation, and estrogen and progesterone levels are also high (Taylor *et al.*, 1998; Taylor *et al.*, 1999).

Furthermore, estrogen and progesterone treatment of endometrial cells *in vitro* is sufficient to increase *HOXA10* and *HOXA11* expression (Taylor *et al.*, 1998; Taylor *et al.*, 1999). However, *in vivo*, 17 β -estradiol suppresses *Hoxa10* expression in the adult mouse uterus, and the synthetic estrogen diethylstilbestrol (DES) suppresses *Hoxa10* in the Müllerian ducts (Ma *et al.*, 1998). One piece of evidence that may explain this suppression comes from a study in which pregnant mice received IP treatment of DES. The reproductive tracts of 2 week old female pups from these mothers displayed posteriorly shifted expression of *Hoxa9*, *Hoxa10*, and *Hoxa11* (Block *et al.*, 2000). This suggests that rather than universally repressing *Hoxa* gene expression, estrogen may regulate the normal positional expression of the *Abd-B*-like *Hoxa* genes *in vivo*.

HOX genes also appear to contribute to epithelial differentiation in non-reproductive tract sites in the adult. Mutations in two of three *Hox* paralogs, *Hoxa9*, *Hoxb9*, or *Hoxd9* do not affect the ability of female mice to become pregnant and deliver healthy offspring, but their mammary glands do not develop properly to lactate sufficiently to feed the pups (Chen and Capecchi, 1999). The post-parturition mammary glands in the mothers exhibit hypoplasia and aberrant morphology in the lobuloalveolar structures, indicating a role for the *Hox9* paralogs in cell proliferation and differentiation in mammary epithelium. Interestingly, *Hox* genes can influence mammary epithelial proliferation and differentiation even when they are expressed by non-epithelial tissue; *Hoxa5* transcript was detected in the stromal, but not in the epithelial compartments of normal mouse mammary glands, yet *Hoxa5* null mice also exhibited lactation defects (Garin *et al.*, 2006). Proliferation in the epithelium of the mammary glands in *Hoxa5*^{-/-}

mice was greatly increased compared to wildtype mice, but, as in the *Hox9* paralog mutants, there were morphological abnormalities in the lobuloalveolar structures.

1.2.3 *HOX* Gene Expression in the Ovary

Very little is known about which *HOX* genes are expressed in the adult ovary, and when they are expressed, and even less is known about *HOX* gene regulation in this tissue. A number of *Hox* genes, including *Hoxa9*, have been shown to be expressed in mouse oocytes (Villaescusa *et al.*, 2004). *HOXA7*, a fellow member of the *HOXA* gene cluster, is expressed in granulosa cells of the human ovarian follicle (Ota *et al.*, 2006). Specifically, *HOXA7* staining was seen in the granulosa cells of growing primary follicles but was absent in the granulosa cells of quiescent primordial follicles, suggesting a potential role for *HOXA7* in cellular proliferation within the ovary. Recently, *HOXA4*, *HOXA7*, and *HOXA9* mRNA have been shown to be expressed in cultured human OSE by real time PCR, and the expression of all three genes decreases as the cultures reach confluence, again suggesting a potential role for certain *HOXA* genes in proliferation (Ota *et al.*, 2009). However, little to no *HOXA7*, *HOXA9*, *HOXA10*, and *HOXA11* immunohistochemical staining is visible in normal human OSE *in vivo* (Cheng *et al.*, 2005).

1.2.4 *HOX* Genes in Cancer

Aberrant expression of an array of *HOX* genes is commonly reported in cancer, and three main possibilities have been identified that characterize this dysregulation

(Abate-Shen, 2002). The first option is that *HOX* genes can be downregulated in a cancerous tissue if it normally expresses that gene. When *HOX* genes are expressed in normal adult tissue, they contribute to the regulation of epithelial differentiation required for proper tissue functioning, such as in the epithelium of the breast and uterus. Therefore, loss of *HOX* gene expression in several different carcinomas is associated with the loss of tissue architecture and organization that is typical of these cancers. For example, loss of *HOXA5* expression is observed in more than 60% of breast cancers (Raman *et al.*, 2000), and *HOXA10* is downregulated in high grade endometrial carcinomas (Yoshida *et al.*, 2006). The second and third possibilities are that *HOX* genes can be re-expressed or overexpressed in tissues that normally express them embryonically, or *de novo* *HOX* gene expression can occur in tissues that have no history of that expression pattern. For example, *HOXA7* and *HOXA9* are critical for the maintenance of the hematopoietic stem cell population, and their overexpression is associated with acute myeloid leukemia (AML) (So *et al.*, 2004). Gain of *HOX* expression has also been demonstrated in many primary solid tumours: *HOXA1* is expressed in neoplastic but not normal mammary tissue (Friedmann *et al.*, 1994), *HOXA1*, *A5*, *A10*, and *HOXC6* are significantly more highly expressed in non-small cell lung carcinoma tissue than in normal lung tissue (Abe *et al.*, 2006), and *HOXA7*, *HOXA9*, *HOXB13* and *HOXD* are upregulated in astrocytic tumours relative to normal astrocytes (Abdel-Fattah *et al.*, 2006).

Just as there are varying *HOX* expression patterns and manners of dysregulation in a wide variety of cancers, there are also many different functional outcomes of aberrant

HOX expression, such as promoting transformation, proliferation, metastasis and invasion. The most fundamental explanation for the abundance of *HOX* gene overexpression in cancer is that they can act as oncogenes, and indeed, this putative *HOX* gene function has been demonstrated in *in vitro* and *in vivo* assays. An early study showed that NIH 3T3 mouse fibroblasts and Rat-1 fibroblasts could be transformed by transfection with *Hoxb4*, as the transfected cells formed colonies in soft agar and tumours in nude mice (Aberdam *et al.*, 1991). Since then, the oncogenic potential of a number of *HOX* genes, including those of the *HOXA* cluster, has been demonstrated; transfection with *Hoxa7* can also transform NIH 3T3 cells (Maulbecker and Gruss, 1993a), and overexpression of *Hoxa9* and its cofactor *Meis1*, or *Hoxa10* in various murine hematopoietic cells types leads to cellular transformation *in vitro* and/or AML *in vivo* (Kroon *et al.*, 1998; Thorsteinsdottir *et al.*, 1997). Notably, epithelium can also be transformed by certain *HOX* genes, as was established by ectopic expression of *Hoxa1* in mammary epithelium (Zhang *et al.*, 2003).

Often, cellular transformation and proliferation go hand in hand, so it is fitting that *HOX* genes have also been shown to play a role in regulating proliferation. Loss of *HOX* function in cancer may be associated with increased proliferation merely as an indirect result of loss of the differentiated state, but there are some studies demonstrating that gain of *HOX* expression has a causal influence on proliferation. For example, overexpression of *HOXB7* increases proliferation via induction of bFGF in a breast adenocarcinoma cell line (Care *et al.*, 1998), and knockdown of *HOXB7* in a human oral carcinoma cell line decreases its proliferation (De Souza Setubal Destro *et al.*, 2010).

Besides the ability of *Hoxb4* to transform rat fibroblasts, it has also been shown that transfection of these cells with *Hoxb4* increases their proliferation relative to the parental cell line (Krosl *et al.*, 1998).

Metastasis and the ability to invade new sites are fundamental components of cancer progression, and *HOX* genes may also play a role in these events. While there are several studies that correlate *HOX* overexpression with invasive cancer types and poor prognosis, there are a few that demonstrate the direct effect of increased *HOX* expression on metastasis and invasion. Namely, knockdown of *HOXB13*, which is overexpressed in endometrial cancer, results in a 90% reduction of the invasive capacity of these cells *in vitro* (Zhao *et al.*, 2005). Of note, since the endometrium is an estrogen-responsive tissue, expression of *HOXB13* in these cells was shown to be induced by 17 β estradiol in a time and dose-dependent manner.

Overall, *HOX* genes have repeatedly been shown to be important players in multiple processes throughout cancer initiation and progression. Characterizing *HOX* gene expression patterns in various cancers and the effects of their gain or loss of function can greatly enhance our understanding of the intracellular pathways guiding neoplastic cellular behaviours, and therefore how to combat them.

1.2.5 *HOXA* Genes in Ovarian Cancer

It has been well documented that aberrant *HOX* gene expression occurs in many cancers, and that gain of function is associated with cellular transformation, increased proliferation, and cancer progression. EOC is no exception to this pattern. Several *HOX*

genes have been shown by microarray studies to be significantly upregulated in ovarian cancer relative to low malignant potential/benign tumours or non-cancerous tissue, including *HOXA7*, *HOXA9* and *HOXA10* (Ota *et al.*, 2007; Bahrani-Mostafavi *et al.*, 2008). In confirmation of these results, expression of these HOXA proteins has also been shown in EOC by immunohistochemistry (IHC), whereas none are expressed in normal OSE (Cheng *et al.*, 2005). Of these genes, the specific involvement of *HOXA10* in ovarian cancer progression has been studied; transfection of ES-2 cells, an ovarian clear cell adenocarcinoma cell line, with *HOXA10* promotes proliferation, migration and invasion (Li *et al.*, 2009). Similar results have been shown with *HOXB13*, another gene that has been shown to be overexpressed in ovarian cancer by real-time PCR (Yamashita *et al.*, 2006; Miao *et al.*, 2007). Targeting endogenous *HOXB13* in ovarian cancer cell lines *in vitro* via antisense introduction or RNA interference resulted in decreased cell proliferation and matrigel invasion, and conversely, infection of a transformed mouse OSE (MOSE) cell line with a retroviral construct expressing *HOXB13* increased its proliferation and rate of tumour formation in nude mice, suggesting that this gene is also involved in EOC progression (Yamashita *et al.*, 2006; Miao *et al.*, 2007).

While it appears that *HOX* genes may play a role in the more traditional aspects of cancer development and progression, they may also contribute to the phenotypically differentiated features of EOC. In particular, *HOXA7* and the *HOXA* genes of the *Abd-B*-like cluster, *HOXA9*, *HOXA10* and *HOXA11* may drive the epithelial differentiation of EOC into its distinct histological subtypes, a unique and previously unexplained feature of ovarian cancer. A report by Cheng *et al.* (2005) showed that the aberrant expression of

the *Abd-B*-like *HOX* genes is subtype-specific; HOXA9 staining was found in serous, endometrioid and mucinous ovarian tumours, HOXA10 stained in endometrioid and mucinous carcinomas but not serous tumours, and HOXA11 was limited to the mucinous subtype. This reflects the expression of *HOX* genes that is seen in the normal reproductive tract tissues mimicked by each subtype, as *HOXA9* is expressed in the oviduct/fallopian tubes and the reproductive tract structures posterior to them, *HOXA10* expression begins in the uterus, and *HOXA11* is only expressed in the posterior uterus and cervix. This normal HOXA protein expression pattern was confirmed in both mouse and human reproductive tract tissues by IHC. Next, this group showed that aberrant expression of particular *HOXA* genes is sufficient to drive histotype-specific differentiation in tumours. When transplanted into nude mice, MOSEC cells, which are spontaneously transformed MOSE cells, form poorly differentiated solid tumours. However, when MOSEC cells are first transfected with murine *Hoxa9*, *Hoxa10*, or *Hoxa11* cDNA and then transplanted into nude mice, the resulting tumours have very distinct histological morphologies bearing resemblance to serous, endometrioid, or mucinous ovarian carcinomas, respectively. This group also confirmed the overexpression of *HOXA7* in EOC that has been previously reported. Interestingly, expression of *HOXA7*, which has been implied but never proven to be integral in Müllerian duct differentiation, was not subtype-specific but was correlated with the degree of differentiation of both the human and mouse ovarian tumours. These data suggest that the unique, histotype-specific epithelial differentiation seen in EOC could be

attributed to increased expression of the *Abd-B*-like *HOXA* genes, and this process is mediated by the concurrent expression of *HOXA7*.

The current understanding of the potential contribution of *HOXA7* to epithelial differentiation of the OSE and EOC is extremely limited and has only been discussed in a few publications. Transfection of the human immortalized OSE cell line IOSE-29 with *HOXA7* was sufficient to change the morphology of these cells from elongated and fibroblastic to a cobblestone morphology that is more typical of epithelial cells (Naora *et al.*, 2001a). It also induced expression of E-cadherin, a marker of epithelial differentiation. However, *HOXA7* may have a more complicated contribution to EOC progression. While there has been no published evidence that ectopic expression of *HOXA7* can transform OSE, *Hoxa7* can transform NIH3T3 cells, but these tumours were histologically characterized as poorly differentiated spindle cell sarcomas (Maulbecker and Gruss, 1993a). This is somewhat comparable to the observation that transfection of MOSEC cells with *Hoxa7* alone did little to alter the morphology of the resulting tumours, and the dramatic epithelial differentiation was only seen when *Hoxa7* was expressed in concert with *Hoxa9*, *Hoxa10*, or *Hoxa11* (Cheng *et al.*, 2005). In addition, a proliferative role for *HOXA7* in the ovary has already been proposed, as it is more highly expressed in the mitotically active granulosa cells of growing follicles than in quiescent primordial follicles (Ota *et al.*, 2006) and in subconfluent OSE than in confluent OSE (Ota *et al.*, 2009). Furthermore, *HOXB7*, a paralog of *HOXA7*, is also more highly expressed in ovarian cancer than in normal OSE, and transfection of immortalized human OSE with *HOXB7* dramatically increased their proliferation rates (Naora *et al.*, 2001b).

In combination, these results suggest that *HOXA7* may contribute to the proliferation, differentiation, and/or transformation of OSE, but the use of various methods and model systems to acquire these results creates a need to investigate these events more comprehensively.

1.2.6 *PAX8* in Ovarian Cancer

PAX8 is another developmental gene that displays many similarities to the *Abd-B*-like *HOX* genes. Like the classical *HOX* genes, *PAX8* is a transcription factor containing a homeodomain, but it also contains a paired domain, which allows for different DNA-binding specificities and interactions with different cofactors than the *HOX* genes (Abate-Shen, 2002). It is expressed in the Müllerian ducts of female embryos and is responsible for epithelial differentiation which is particularly well established in thyroid and kidney developmental studies (Mansouri *et al.*, 1996). *PAX8* is also expressed in adult tissues, including normal fallopian tube epithelium, ovarian inclusion cysts, and several subtypes of ovarian cancer, especially the serous subtype (Bowen *et al.*, 2007). Notably, like the *HOX* genes, *PAX8* is not expressed in normal human OSE (Bowen *et al.*, 2007).

Not only is *PAX8* found in established human ovarian cancers, but it might also be capable of driving metaplasia in certain cell types, which is a required step in the malignant transformation of OSE. One group recently found that *PAX8* is more highly expressed in the peritoneum of women with endometriosis compared to those without endometriosis, and they showed by *in situ* hybridization that *PAX8* is aberrantly expressed in apparently normal peritoneum without any signs of preexisting

endometriosis or endosalpingiosis (Gaetje *et al.*, 2007). This lead them to suggest that endometriosis might be caused in some cases by *PAX8*-driven metaplasia of peritoneal cells to become an endometriotic lesion, though this claim has yet to be investigated in more mechanistic detail. In addition, the oncogenic potential of the *PAX* gene family has been demonstrated by showing that NIH 3T3 and 208 cells (subcloned from Rat-1 fibroblast cells) transfected with *Pax8* form colonies in soft agar and tumours in nude mice (Maulbecker and Gruss, 1993b). More recent work has provided evidence to support the hypothesis that *PAX8* plays an integral role in cancer cell survival, as *PAX8* has been shown to inhibit transcription of the tumour suppressor p53 (Stuart *et al.*, 1995) and can activate transcription of the anti-apoptotic gene *BCL2* (Hewitt *et al.*, 1997). How *PAX8* contributes to the differentiation or any other aspect of EOC has not been clearly established, but the fact that its expression patterns in normal tissues and EOC so closely mirror those of the *HOXA* genes warrants its investigation.

1.3 Transgenic Mouse Models of Ovarian Cancer

1.3.1 The tgMISIIR-TAg Mouse Model

Mouse models of ovarian cancer are valuable experimental tools in which to study the early events and progression of EOC. The first transgenic mouse model, the tgMISIIR-TAg model of EOC, was described by Connolly *et al.* in 2003. This model utilizes the Müllerian inhibitory substance type II receptor (MISIIR) promoter to drive expression of the early region of Simian virus 40 large and small T antigens (TAg) (**Figure 3**). The *MISIIR-TAg* transgene was injected into fertilized eggs from B6C3F1

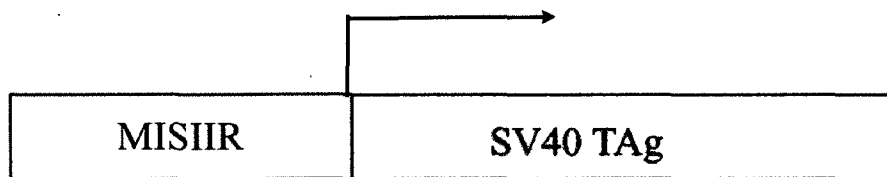


Figure 3: The tgMISIIR-Tag transgene construct. The expression of the potent oncogene SV40 TAg is driven by the MISIIR promoter in the epithelium of the female reproductive tract and the OSE.

mice (from a C57BL/6 and C3H cross), and 50% of the transgene-bearing founder mice developed poorly differentiated epithelial ovarian tumours with regions of papillary or glandular differentiation by 91 days of age. The ovarian tumours express cytokeratins 8 and 19, but not α -inhibin, indicating that they were epithelial and not granulosa or sex-cord stromal tumours. The Vanderhyden lab has established a line of tgMISIIR-TAg mice using the same transgene construct but on the FVB/N background. 100% of female mice develop poorly differentiated bilateral ovarian tumours with a prenatal onset and average endpoint of 105 days of age, and the tumours express α -inhibin in addition to cytokeratins 8 and 19 (Vanderhyden lab, unpublished data). One cell line derived from the ovarian tumour of a tgMISIIR-TAg mouse was used for this project, which will be referred to as the MISIIR-TAg cell line.

1.3.2 The tgCAG-LS-TAg Mouse Model

While the tgMISIIR-TAg model has proven to be very useful and efficient, the animals start to develop nascent ovarian tumours before they are born, which does not reflect the postmenopausal onset of EOC that is commonly seen in humans. To address this issue and to control the time of tumour onset, our lab has developed and recently described a novel, surgically inducible transgenic mouse model of epithelial ovarian cancer called the tgCAG-LS-TAg mouse model of ovarian cancer (Laviolette *et al.*, 2010). The DNA of these mice is arranged such that LoxP sites flank a β -geo gene (a fusion of the β -galactosidase and neomycin phosphotransferase genes) and a stop sequence preceding *TAg* (**Figure 4A**). If left untreated, these mice express β -

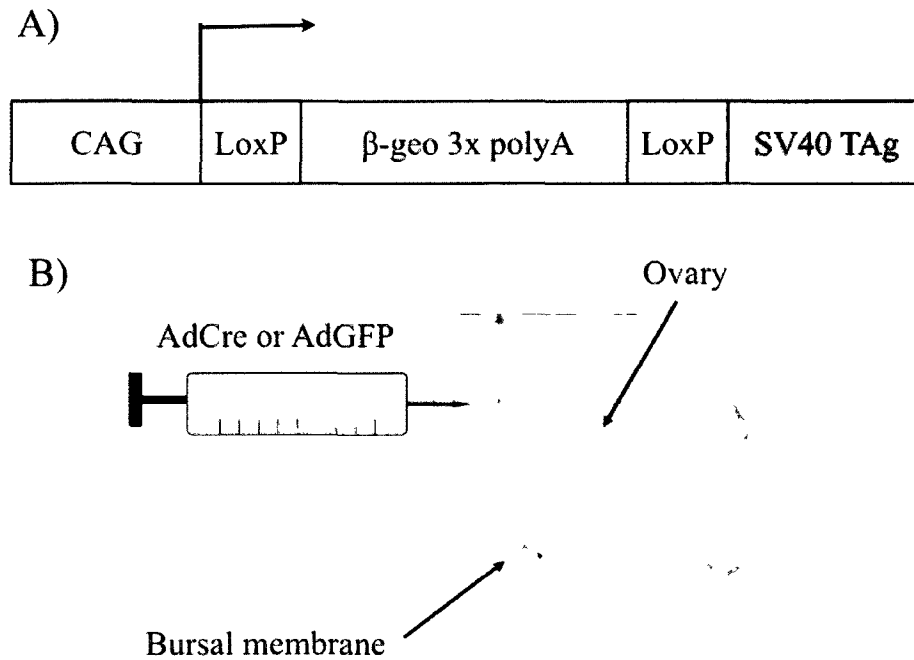


Figure 4: Induction of TAg expression in tgCAG-LS-TAg mice. (A) The CAG-LS-TAg transgene construct. The ubiquitous CAG promoter drives the expression of β -galactosidase in all tissues of untreated mice. AdCre recognizes the LoxP sites and mediates the deletion of the β -geo/3xpolyA cassette, leading to the expression of SV40 TAg. (B) AdCre or the control virus AdGFP are injected under the ovarian bursa, exposing the OSE to virus but preventing viral spread to other organs.

galactosidase in all tissues driven by the ubiquitous cytomegalovirus early enhancer/chicken β -actin (CAG) promoter. However, in the presence of Cre recombinase, the DNA between the LoxP sites is excised by way of Cre-mediated recombination, and TAg is expressed. To limit TAg expression and therefore tumourigenesis to the OSE, an adenovirus expressing Cre recombinase (AdCre) is injected under the ovarian bursa, a membrane surrounding the mouse ovary that can be used as a physical barrier to virus spread and subsequent TAg expression (**Figure 4B**). 82% of the tgCAG-LS-TAg mice treated with AdCre developed ovarian tumours with a median survival time of 113 days post-surgical induction of TAg. None of the animals injected with the control adenovirus expressing green fluorescent protein (AdGFP) developed ovarian tumours or died before their scheduled endpoint at 365 days post-surgery. Most of the tgCAG-LS-TAg animals also developed ascites, which is an accumulation of fluid in the peritoneum containing tumour cells, and an exceedingly common component of the human disease.

The role of steroid hormones was also investigated in the tgCAG-LS-TAg model. After surgical injection of either AdGFP or AdCre, the mice were treated with a control, 17 β -estradiol (E2), or progesterone (P4) pellet that continually released hormone into the bloodstream for 60 days. Some of the tumours from the mice treated with estrogen exhibited areas of papillary differentiation, which was not seen in the control tumours. The evidence that *HOXA* genes play a role in transformation and differentiation of human ovarian cancers raises the question whether *Hoxa* genes might be responsible for the altered differentiation statuses of these mice. Three ascites cell lines derived from the tgCAG-LS-TAg animals injected with AdCre, one from each treatment group, were used

for this study. These lines are referred to as MASC2 (mouse ascites control 2), MASE2 (mouse ascites estrogen 2) and MASP1 (mouse ascites progesterone 1).

1.4 Project Rationale

While ovarian tumours from both transgenic mouse models are epithelial in nature and express cytokeratins, they are generally of a poorly differentiated histology and do not recapitulate the histotypes of human EOC. In addition, cell lines derived from these models usually do not exhibit the classical cobblestone morphology indicative of a differentiated epithelium. It is vital that animal models of disease are representative of the human condition on the histological and genetic level so that they may be used to enhance our understanding of disease etiology and serve as a platform for testing novel therapeutics. We initially wanted to characterize the endogenous expression levels of *Hoxa7*, *Hoxa9*, *Hoxa10*, *Hoxa11*, and *Pax8* to determine if, as in human EOC, there were any differences in the expression of these genes in normal MOSE compared to tumourigenic cell lines and tumours. Since there is mounting evidence that increased *HOXA* and *PAX8* gene expression in human EOCs may play a significant part in their differentiation, we wanted to see if this theory could be applied to our mouse models of ovarian cancer, and if we could enhance the histological differentiation of these tumours as a result.

Overexpressing *Hoxa7* in cell lines from these models arose as a method of attempting to initially achieve this differentiation *in vitro*. If *Hoxa7* demonstrated the capacity to enhance the epithelial morphology of the mouse ovarian cancer cells *in vitro*,

these cells could then be xenografted into severe combined immunodeficiency (SCID) mice to see if this differentiation could be maintained in tumours. This would also establish if manipulating *Hoxa7* expression in our two mouse models of EOC could be an effective way of making those tumours more histologically similar to human ovarian tumours. We also proposed to determine if *Hoxa7* is a factor in the infrequent but striking appearance of papillary structures in tgCAG-LS-TAg mice treated with estrogen. While it is currently unknown whether *Hoxa7* is regulated by estrogen, given the unique ability of *Hoxa7* to increase differentiation in ovarian tumours, which develop from an estrogen-responsive tissue, we hypothesized that E2 enhances *Hoxa7* expression to mediate the increase in differentiated features. And finally, since *Hoxa7* is expressed in ovarian inclusion cysts, has some *in vitro* transformative capacity and is associated with mitotically active cells within the ovary, we wanted to examine the ability of *Hoxa7* to transform normal MOSE or increase proliferation in order to understand its potential contribution to ovarian cancer initiation and progression.

1.5 Hypothesis

We hypothesized that our cancer cell lines and tumours derived from mouse models of poorly differentiated EOC would not endogenously express high levels of the *Hoxa* or *Pax8* genes, and that overexpression of *Hoxa7* in these cell lines would change their proliferation rates, promote their epithelial differentiation *in vitro*, and transform normal MOSE.

1.6 Project Objectives

- 1) To characterize the proliferation rates of normal MOSE, and in MOSE-RM, tgCAG-LS-TAg and tgMISIIR-TAg derived cell lines
- 2) To characterize the endogenous expression levels of *Hoxa7*, *Hoxa9*, *Hoxa10*, *Hoxa11* and *Pax8* in normal MOSE and in MOSE-RM, tgCAG-LS-TAg and tgMISIIR-TAg derived cell line
- 3) To induce overexpression of *Hoxa7* in all cell lines and determine its effect on normal MOSE and ovarian cancer cells *in vitro* by examining:
 - proliferation rates
 - morphology in culture
 - expression of epithelial and mesenchymal markers
 - colony forming ability of *Hoxa7*-transduced MOSE
- 4) To compare the expression levels of *Hoxa7* and/or *Pax8* in cell lines to ascites and tumour samples from our transgenic mouse models

CHAPTER 2: MATERIALS AND METHODS

2.1 Tissue Culture

2.1.1 Isolation of Normal MOSE and Mouse Ovarian Cancer Cells

The following cell lines were used in this project: normal MOSE, MOSE-RM, MASC2, MASE2, MASP1, and MISIIR-TAg. The origins of the cell lines are listed in **Table 1**. Primary MOSE cells were isolated from surgically isolated ovaries from 6-8 week old FVB/N mice (Charles River Laboratories, Wilmington, MA). The animals were euthanized by carbon dioxide asphyxiation and the ovaries were removed aseptically, with complete removal of the bursal membrane. The ovaries were placed in phosphate buffered saline (PBS; HyClone, Logan, UT) and washed three times, then transferred to 15 ml Falcon tubes (Becton Dickinson, Franklin Lakes, NJ) containing PBS with 0.25% trypsin (Hyclone). Tubes were placed in a 37°C incubator for 40 minutes, then inverted three times to mechanically remove any epithelial cells on the surface of the ovary. The supernatant containing these cells was centrifuged at 1000 rotations per minute (rpm) for 10 minutes. The cell pellet was resuspended in fresh MOSE media [α -minimum essential medium (α -MEM; Hyclone) supplemented with 4 or 10% heat-inactivated 3:1 donor bovine serum:fetal bovine serum (DBS:FBS; PAA Laboratories Inc., Etobicoke, ON), 1.8×10^{-3} μ g/ml EGF (Roche, Indianapolis, IN), 5 U/ml penicillin/ 0.005mg/ml streptomycin solution (Sigma-Aldrich, Oakville, ON), 0.1 μ g/ml gentamicin (Invitrogen, Grand Island, NY) and insulin-transferrin-sodium-selenite solution (ITSS; 5 μ g/ml insulin, 5 μ g/ml transferrin, 5 ng/ml sodium selenite, Roche)] and plated.

The MOSE-RM cell line was derived from the transgenic ImmortoMouse, which expresses a temperature-sensitive mutant large TAg driven by the ubiquitous H-2K^b

Cell Line	Origin	Tumourigenic?
MOSE	Normal mouse ovarian surface epithelium	No
MOSE-RM	Immortalized MOSE cells derived from “Immortomouse” expressing temperature-sensitive TAg (Yao <i>et al.</i> , 2006) + retroviral infection of <i>K-ras</i> ^{G12D} and <i>c-myc</i>	Yes
MASC2	Derived from the ascites of a tgCAG-LS-TAg mouse treated with a control pellet after activation of the TAg oncogene	Yes
MASE2	Derived from the ascites of a tgCAG-LS-TAg mouse that was treated with estrogen pellet after activation of the TAg oncogene	Yes
MASP1	Derived from the ascites of a tgCAG-LS-TAg mouse that was treated with a progesterone pellet after activation of the TAg oncogene	Yes
MISIIR-TAg	Derived from the tumour of a tgMISIIR-TAg mouse	Yes

Table 1: MOSE and mouse ovarian cancer cell lines that were used in this study.

promoter. MOSE from these female mice are immortalized but not transformed when maintained at 32°C, which is the permissive temperature for TAg expression. These “ImmortoMOSE” were isolated in the same manner as normal MOSE, and were transformed by our lab by retroviral infection with two oncogenes, *K-ras*^{G12D} and *c-myc*, and are referred to as MOSE-RM cells. The MOSE-RM cells proliferate faster than ImmortoMOSE *in vitro*, and unlike the ImmortoMOSE, they can invade through Matrigel, and form colonies in soft agar and poorly differentiated tumours in nude mice (Yao *et al.*, 2006).

The MASC2, MASE2, and MASP1 cell lines were derived from ascites cells collected from ovarian tumour-bearing tgCAG-LS-TAg mice. While the tumours were forming, the mice were treated with a control pellet, a 60-day release estrogen pellet, or a 60-day release progesterone pellet that had been surgically implanted subcutaneously; the resulting ascites cell lines were named for the treatments the mice received: mouse ascites control 2 (MASC2), mouse ascites estrogen 2 (MASE2), and mouse ascites progesterone 1 (MASP1). At necropsy, the ascites were needle aspirated from the peritoneal space and placed directly in culture with MOSE media supplemented with an extra 0.1 µg of gentamicin and 0.26 U of penicillin/streptomycin. The media with extra antibiotics was replaced the next day, and cells were maintained with regular MOSE media with 10% DBS:FBS after a few passages.

The MISIIR-TAg cell line came from cells isolated from the right ovarian tumour of a tgMISIIR-TAg mouse. The tumour was removed, diced and strained through a microfilter with MOSE media into a 50 ml Falcon tube, and the tube was centrifuged for 4 minutes at 4000 rpm. The cell pellet was resuspended in fresh MOSE media

supplemented with an extra 0.1 µg of gentamicin and 0.26 U of penicillin/streptomycin and plated. After 4 passages in culture, the tumour cells were maintained in regular MOSE media with 10% DBS:FBS.

2.1.2 Cell Maintenance

All murine cell lines were maintained in MOSE media with 10% DBS:FBS. The human ovarian cancer cell line A2780s (source: M. Molepo, original reference: Eva *et al.*, 1982) was used as a positive control in the colony formation assays, and these cells were cultured in Dulbecco's minimum essential media (D-MEM; HyClone) with 10% DBS:FBS. Cell cultures were maintained in an incubator at 37°C equilibrated with 5% CO₂.

Unless specified otherwise, cells were incubated in 75 cm² flasks (Corning Inc., Corning, NY) containing 10 ml of either MOSE media or D-MEM + 10% DBS:FBS until they reached confluence, at which point they were passaged. Cells were first washed with phosphate-buffered saline (HyClone) and incubated for 5-10 minutes with 0.05% trypsin (HyClone). When cells had detached from the flask, the trypsin was inactivated with media, cells were resuspended, and plated in a new flask at a dilution of 1:10-1:20. Cells were frozen and stored at -80°C in 3:1 DBS:FBS with 10% dimethylsulfoxide (DMSO; Fisher-Scientific, Fair Lawn, NJ) in Cryoware™ Cryovials (Nalge Nunc International Corp., Rochester, NY). Cells were thawed at 37°C and transferred immediately to 25 cm² flasks (Corning) containing 5 ml of either MOSE media or D-MEM + 10% DBS:FBS. All experimental cell lines were tested for and confirmed to be free from mycoplasma contamination.

2.1.3 Proliferation Assays

At 70-100% confluence, all MOSE and ovarian cancer cell lines were washed with PBS, trypsinized, and resuspended in 10 ml of MOSE media. Cells from each line were counted using the Vi-CELL™ XR Cell Viability Analyzer (Vi-CELL; Beckman Coulter Inc., Brea, CA) and plated at a density of 2×10^4 cells per well in 6-well plates (Corning) with 2 ml of MOSE media. Three wells per cell line were counted at 24, 48, 72, and 96 hours post-plating. At each time point, the media was aspirated from 3 wells, cells were washed with PBS, incubated in trypsin for 5 min, and resuspended in 1 ml of MOSE media. These cell suspensions were counted using the Vi-CELL counter. The total number of viable cells was used for all calculations. The media was changed in the 72 and 96 hour cultures at 48 hours post-plating. Three replicates of the proliferation assay were performed for each cell line.

2.1.4 Assessment of Morphology

The morphology of cultured cells was established by visual examination, and photographs were taken using a Coolpix 5400 digital camera (Nikon Instruments Inc., Melville, NY) mounted on the Eclipse TE2000-U inverted light microscope (Nikon Instruments Inc.). Cells were characterized based on exhibition of classically defined epithelial characteristics (including a cobblestone phenotype and the formation of cell rafts) or mesenchymal characteristics (such as fibroblastic projections).

2.1.5 Treatment of Cells with EGF

The effect of EGF on *Hoxa7* expression in cells *in vitro* was assessed in two different experiments. In the first, all cell lines were brought back from frozen vials by thawing at 37°C and maintained in normal MOSE media (containing EGF, described previously) for 2-3 passages, then split into MOSE media containing EGF (EGF+ media) or MOSE media without EGF (EGF- media). Cells (now referred to as EGF+ or EGF- cells) were maintained in their respective media for 3-4 passages, then trypsinized and pelleted for RNA extraction, cDNA preparation and *Hoxa7* expression analysis by quantitative-PCR (Q-PCR; n = 2). In the second experiment, EGF+ and EGF- cells that had already been established from the first experiment were plated in 6-well dishes, 6 wells with EGF- cells and 3 wells with EGF+ cells. Each cell line received its appropriate media. For each cell line, an appropriate density at which to plate the cells was chosen so that the wells would be 70-100% confluent after 72 hours in culture; however, within each cell line, the EGF+ and EGF- cells were plated at the same density (3x10⁵ cells per well for the MOSE-RM, MASC2 and MASP1, 4x10⁵ cells per well for the MASE2, 5x10⁵ cells per well for the MOSE, and 8x10⁵ cells per well for the MISIIR-TAg cells). At 24 hours post-plating, the media was changed on all cells. Three wells of EGF- cells received EGF- media, 3 wells of EGF- cells received EGF+ media (then called EGF-/++ cells), and the 3 wells of EGF+ cells received EGF+ media. At 48 hours after the media change (72 hours after plating), all cells were trypsinized and pelleted for RNA extraction and cDNA was made for subsequent Q-PCR analysis of *Hoxa7*.

2.1.6 Ascites Cell Growth in Suspension

Autoclaved agarose (1%) was melted in a microwave and 6 ml was added to bacterial grade plates (Fisher-Scientific). When the agarose had solidified, 8-10 ml of MOSE media was added to each plate and placed in the 37°C incubator for 2-3 hours to equilibrate. After this time, the media was aspirated and 10 ml of fresh media was added and trypsinized ascites cell suspensions in media were added to these plates. 5×10^5 MASC2 and MASP1 cells, and 1×10^6 MASE2 cells suspended in media were added to the agarose plates to force them to grow in suspension. At the same time, $3-4 \times 10^4$ cells of each cell line were also plated in duplicate in 6-well dishes to compare the same passage of cells in normal tissue culture attachment conditions. Two to three days after adding the cell suspensions to the agarose, the presence of cell spheres/clusters was confirmed under the microscope and pictures were taken. All the media and cells were collected in 15 ml Falcon tubes and centrifuged at 3000 rpm for 3-5 minutes in a clinical centrifuge (International Equipment Co., Chattanooga, TN), at which time the media was aspirated from the cell pellet. The cell pellet was washed with PBS and then centrifuged again, the PBS was aspirated and 500 µl of trypsin was added to disaggregate the cell clusters. After 2-5 minutes in trypsin, 500 µl of media was added to the trypsin and cells. A 100 µl aliquot of the cell suspension was added to 900 µl of media to count a 10X dilution of cells and confirm their viability in the Vi-CELL counter (Beckman-Coulter). The rest of the cell suspension was centrifuged again, the media was aspirated, and pellets were either used right away for RNA extraction or snap frozen in dry ice and isopropanol and stored at -80°C for later use. RNA was extracted from all the cell pellets, cDNA was made by reverse transcription polymerase chain reaction (RT-PCR), and all samples were

assessed for *Hoxa7* expression by Q-PCR. This experiment was completed three times for both the suspension and attachment conditions.

2.2 RNA Analysis

2.2.1 Extraction and Quantification of RNA

At 100% confluence in 75 cm² flasks (approximately 1×10^7 cells), cells were washed, trypsinized, and resuspended in 5-10 ml MOSE media. Cell suspensions were transferred to 15 ml Falcon tubes and spun down at 3000 rpm for 3-5 minutes in a clinical centrifuge (International Equipment Co.). The media was aspirated and cells were resuspended in 1 ml of PBS to wash them. Cells were spun down again at 3000 rpm for 3-5 minutes and the PBS was aspirated. At this point, either RNA was extracted from the cell pellets immediately, or pellets were snap frozen for 5 minutes in an isopropanol and dry ice bath and stored at -80°C for future use.

Total RNA was extracted using the RNeasy Mini-Kit (Qiagen, Mississauga, ON). Fresh cell pellets were kept on ice and homogenized immediately, and frozen cell pellets were thawed on ice for 5 minutes to loosen the cell pellet before homogenization. Cell pellets were suspended in 600 µl RLT buffer (Qiagen). Homogenization was performed using either a 25 gauge needle and 1 ml syringe or QIAshredders (Qiagen). Samples were processed through multiple wash/spin cycles according to the RNeasy protocol and total RNA was eluted from the spin column in either 30 µl or 50 µl of RNase-free water. The RNA was treated with DNase in 10X DNase buffer (Ambion, Austin, TX) to eliminate any remaining contaminating DNA and incubated in a 37°C waterbath for 30 minutes.

The DNase was inactivated with 10X DNase inactivator reagent (Ambion) at room temperature for 5 minutes, then spun down at $\geq 10,000$ rpm for 1 minute. The RNA-containing supernatant was collected and transferred to a new tube. Total RNA was quantified by measuring the optical density of 1 μ l of RNA using the NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Waltham, MA). RNA samples were stored at -80°C until needed.

2.2.2 Reverse Transcription-PCR

cDNA was made from 500 ng total RNA from each RNA extraction by RT-PCR. The appropriate volume of sample RNA to give 500 ng was added to each PCR tube and brought to a final volume of 11 μ l with ddH₂O. 1 μ l of 300 ng/ μ l random primers (Invitrogen; all RT-PCR reagents from Invitrogen unless specified otherwise) and 1 μ l of 10 mM mixed deoxynucleotide triphosphates (dNTPs) were added to the RNA/H₂O solution. The RNA tubes were incubated at 65°C for 5 minutes and then at 4°C for 1 minute. Each sample was then brought to a final volume of 20 μ l with 4 μ l 5X First Strand Buffer, 1 μ l dithiothreitol (DTT), 1 μ l RNase Out, and 1 μ l (200 U) of Superscript RT III, and incubated at 25°C for 5 minutes, 50°C for 50 minutes, and either 70°C for 15 minutes or 85°C for 5 minutes. Genomic DNA contamination was excluded in each PCR reaction using a control that was generated without reverse transcriptase, and one that was generated without RNA. cDNAs were stored at -20°C .

2.2.3 Quantitative-PCR

Oligonucleotide primers for Q-PCR were designed using the Invitrogen OligoPerfect™ primer design software to amplify murine *Hoxa7*, *Hoxa9*, *Hoxa10*, *Hoxa11* and *TAg*, and custom ordered from Invitrogen. The endogenous control gene *18S* was amplified in all reactions as a reference for mRNA quantification. Several primer sets for each gene were ordered and optimized. The primer sequences were as follows: *Hoxa7* (5'-GTC AAC AGC CCC CTC TAT CA-3' and 3'-CGT CAG GTA GCG GTT GAA AT-5'), *Hoxa9* (5'-TAG AGA AAA ACA ACC CAG CGA AG-3' and 3'-CAG CGT CTG GTG TTT TGT GTA A-5'), *Hoxa10* (5'-AGC TCG CTA GTC CCT TTC CT-3' and 3'-GAT TCG GTT TTC TCG GTT CA-5'), *Hoxa11* (5'-CTC CAG CCT CCC TTC TTT TT-3' and 3'-GGC TCA ATG GCG TAC TCT CT-5'), *TAg* (5'-GGT GGG TTA AAG GAG CAT GA-3' and 3'-TAG TGG CTG GGC TGT TCT TT-5'), and *18S* (5'-CGC GGT TCT ATT TTG TTG GT-3' and 3'-AGT CGG CAT CGT TTA TGG TC-5'). For the Q-PCR reactions, all sample cDNAs were diluted 1:10 for the *Hoxa* primers and 1:10,000 for the *18S* primers. Each well contained 2 µl cDNA in a total volume of 20 µl per reaction, in triplicate on a 96-well plate (Applied Biosciences, Foster City, CA). Each well also contained 6 µl H₂O, 1 µl of each primer pair (at 10 µM for each primer), and 10 µl of 2X SYBR® Green PCR Master Mix, which contains the fluorescent SYBR® Green dye, AmpliTaq Gold® DNA polymerase, and the required buffers (Applied Biosciences).

The absolute Q-PCR method was utilized, which compares the amplification of sample cDNA to that of a standard curve to produce an absolute quantity of cDNA per reaction. Standards for each primer set were generated by amplifying cDNA from a positive control for each primer set using the relative Q-PCR method. 5 µl of the PCR

products were run on a 1% agarose gel at 100V next to 5 µl of the MassRuler™ Low Range DNA Ladder (Fermentas, Burlington, ON) and digital images of the gels were taken with the EpiChemi II Darkroom and Bioimaging Systems (UVP, Upland, CA). The density of the PCR product bands were compared to the standardized DNA densities in each band of the MassRuler™ DNA Ladder and quantified using the AlphaEaseFC software (Cell Biosciences, Inc. Santa Clara, CA). A range of serial dilutions was then prepared for each standard which was tested by Q-PCR with some of the experimental cDNA samples to ensure that an appropriate range of concentrations was used for each primer standard set. Five 10-fold serial standard dilutions were run in triplicate on each Q-PCR plate, for each primer set being tested on that plate. The efficiency of the standard curve for each primer set was calculated using the online Stratagene QPCR Standard Curve Slope to Efficiency calculator, which uses the formula: $\text{efficiency} = -1 + 10^{(-1/\text{slope})}$. Each standard curve for all primer sets yielded a slope within the accepted range of -3.1 to -3.6 and a greater than 90% efficiency.

Q-PCR was carried out using the the ABI 7500 Real-Time PCR System (Applied Biosciences). The reaction conditions were as follows: AmpliTaq Gold® DNA polymerase activation (95°C for 10 minutes), 40 PCR cycles of denaturation (95°C for 15 seconds) and annealing (*Hoxa7* and *18S* plates at 65°C, and all other primer sets and *18S* plates at 60°C for 1 minute), and dissociation (95°C for 15 seconds, 60°C for 1 minute, 95°C for 15 seconds). Fluorescence data was collected at the annealing stage of each cycle. Since SYBR Green is a non-specific, double stranded DNA binding dye, any Q-PCR product would have contributed to the measured fluorescence. To exclude the possibility of quantifying non-specific amplification, including primer dimers, the

dissociation stage was performed for every Q-PCR. During this step, the temperature is raised and the corresponding drop in fluorescence is detected as the two strands of amplified cDNA dissociate. The rate of change of fluorescence with respect to time is plotted on the dissociation curve, and the presence of a single peak on the curve (**Figure 5A**) represents the single, specific amplicon of interest. The PCR product sizes were as follows and were confirmed by separating the products on a 1% agarose gel at 100V: *Hoxa7* (297 bp), *Hoxa9* (171 bp), *Hoxa10* (105 bp), *Hoxa11* (126 bp), *Pax8* (263 bp), *TAg* (194 bp), *18S* (219 bp). The 1 Kb Plus DNA Ladder (Invitrogen) was also run on the gels to determine band size (**Figure 5B**). There were no detectable bands in the no template and no reverse transcriptase control lanes, indicating the absence of genomic DNA contamination in the reactions. The Q-PCR results were assessed using the ABI 7500 SDS software (Applied Biosystems). The absolute quantity of the gene of interest for each sample was normalized to that sample's expression of the endogenous control *18S*.

2.3 Lentiviral Transduction

2.3.1 Lentiviral Constructs

The cDNA for murine *Hoxa7* (accession number BC036986, clone ID 4986801) was purchased from OpenBiosystems (Thermo Scientific, Huntsville, AL) and sequenced to compare to the Consensus Coding Sequence derived from the genomic sequence in the CCDS database. This clone was found to be missing the N-terminal 35 amino acids, but this region is homologous to the human *Hoxa7* gene. The human Incyte clone for *Hoxa7*

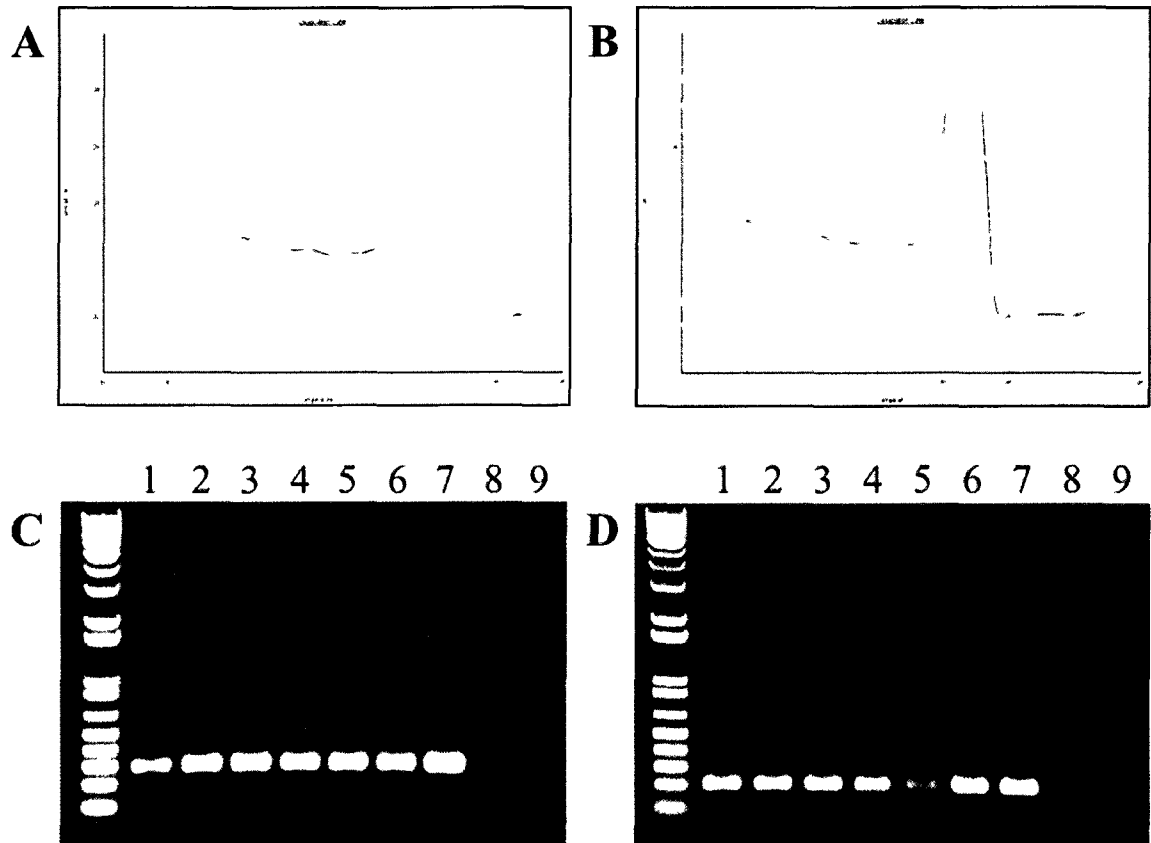


Figure 5: *Hoxa7* and *18S* dissociation curves and confirmation of band sizes.

Representative dissociation curves for amplification of *Hoxa7* (A) and *18S* (B) in the oviduct, which was used as a positive control for *Hoxa7* expression. A dissociation curve was run for each Q-PCR plate to confirm the amplification of a single product without primer dimer. Amplicons at the correct band size for each primer set were also confirmed, and shown here are those for *Hoxa7* (C; 297 bp) and *18S* (D; 219 bp) in the following samples: MOSE (1), MOSE-RM (2), MASC2 (3), MASE2 (4) MASP1 (5), MISIIR-TAg (6), oviduct (7) H₂O (no template) control (8), RT- control (9).

cDNA was purchased (clone ID LIFESEQ3152180) and the sequence homology to that of the murine *Hoxa7* at the region of the N-terminal 35 amino acids was confirmed. A PCR cloning strategy was utilized to create a full-length recombinant murine *Hoxa7* cDNA that was subcloned into the lentiviral vector pWPI (obtained from Dr. Didier Trono, École Polytechnique Fédérale de Lausanne, Switzerland). *Hoxa7* was inserted between the ubiquitous promoter EF1- α and the internal ribosome entry site (IRES), which links the expression of *Hoxa7* to that of the downstream GFP gene (**Figure 6**). This construct will be referred to as *Hoxa7*-LV. The lentivirus (LV) pWPI was also used as a control virus in all transduction experiments, and will be referred to as WPI-LV.

2.3.2 Lentiviral Production

The WPI-LV and *Hoxa7*-LV were produced by transfecting 293T cells (obtained from Dr. Doug Gray's lab, OHRI, Ottawa, ON) with 20 μ g of the viral vector DNA, 15 μ g of the packaging plasmid pCMVdR8.74 (Trono lab), and 6 μ g of the envelope plasmid pCAG-Eco (obtained from Dr. Arthur Nienhuis, Memphis, TN; Hanawa *et al.*, 2002). H₂O, 250 μ l of 0.5 M CaCl₂, and 500 μ l of 2X HeBS buffer were mixed with the DNA and plasmid components while vortexing, then incubated at room temperature for 30 minutes. This mixture was added drop-wise to 10 cm plates that had been seeded 24 hours previously with 2-2.5x10⁶ 293T cells. Cells were incubated overnight and the media was aspirated and replaced with 6 ml of fresh media 24 hours after transfection. Another 24 hours later, cells were examined for GFP expression under a fluorescent microscope to assess transfection efficiency; approximately 80-90% of all transfected

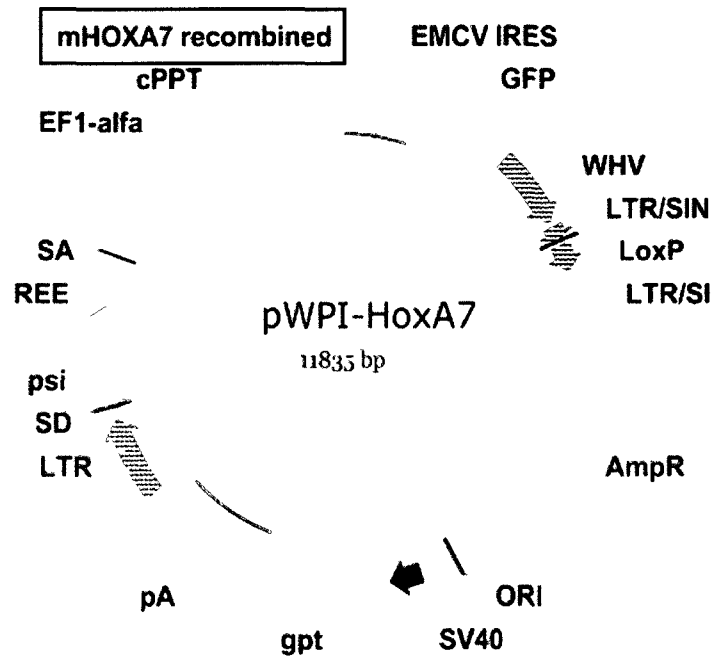


Figure 6: *Hoxa7* lentiviral vector map. The murine *Hoxa7* gene was subcloned into the bicistronic lentiviral vector pWPI, between the ubiquitous EF1- α promoter and the IRES. Successful infection and *Hoxa7* expression in the cells is indicated by the expression of GFP, which follows the IRES. The pWPI backbone was used as the vector control, referred to as WPI-LV.

cells were green. The media containing the released virus produced by the confluent 293T cells was collected, spun for 5 minutes at 3000 rpm at room temperature, then filtered (0.45 μ M). The media-virus solution was snap frozen in isopropanol and dry ice and stored at -80°C for future use.

2.3.3 Lentiviral Transduction of Cell Lines

Overexpression of *Hoxa7* was induced in all cell lines listed in Table 1 by transduction with the *Hoxa7*-LV. All cell lines were also infected with the WPI-LV alone as a control for transduction. $2-4 \times 10^5$ cells were plated in 6-well dishes. The media was aspirated 24 hours later and replaced with 400-500 μ l of either WPI-LV or *Hoxa7*-LV + 6 μ g/ml Polybrene (Sigma) per well and the plates were spun at 1800 rpm at 22°C for 45 minutes. At this time, the virus was aspirated and fresh virus was applied. Plates were spun again at 1800 rpm at 22°C for 45 minutes, then 2 ml fresh MOSE media was added to the wells and the plates were incubated. Most cell lines were transduced again with the appropriate virus within one week of initial transduction.

2.3.4 Confirmation of Transgene Expression

All cell lines were maintained as a mixed population of infected and uninfected cells over a period of several weeks. In the *Hoxa7*-LV bicistronic vector, *Hoxa7* is linked to GFP via an IRES, meaning that GFP expression can be used as an indirect measure of successful transduction. The mixed populations of cells treated with the WPI-LV and the *Hoxa7*-LV were assessed for GFP expression by flow cytometry using the EPICS XL

Flow Cytometer (Beckman Coulter). Cells were trypsinized and 1×10^6 cells were resuspended in 1 ml PBS + 2% serum for this procedure. Doubling times of the GFP-positive and negative fractions of transduced MOSE-RM, MASC2, MASE2 and MASP1 cells in the mixed populations were calculated using the equation: doubling time = $(t_2 - t_1)/y$, where y = the number of doubling times, t_2 = time 2 and t_1 = time 1. The number of doubling times (y) was calculated using the equation $y = \log(X_2/X_1)/\log 2$, where X_2 = number of cells at time 2 and X_1 = number of cells at time 1.

For each cell line, an untransduced control, WPI-LV-transduced, and *Hoxa7*-LV-transduced samples of cells were sorted by FACS MoFlo Cell Sorting by Paul Oleynik (OHRI, Ottawa, ON). GFP-positive cells were collected and placed in culture. WPI-LV-transduced, sorted GFP-positive cells will be referred to as WPI cells. *Hoxa7*-LV-transduced, sorted GFP-positive cells will be referred to as *Hoxa7* cells. GFP expression in WPI cells and *Hoxa7* cells was confirmed by fluorescence imaging with the Axioskop 2 MOT fluorescent microscope (Carl Zeiss Canada Ltd. Toronto, ON). Overexpression of *Hoxa7* in the *Hoxa7* cells was confirmed by Q-PCR.

2.4 Assessment of Lentivirally-transduced Cells

2.4.1 Proliferation Assays

Proliferation assays for WPI cells and *Hoxa7* cells in all experimental cell lines were performed in triplicate in the same manner as the proliferation assays for the untransduced cells (see section 2.1.3).

2.4.2 Assessment of Morphology

Hoxa7 cells were examined for any changes in morphology relative to WPI cells or untransduced cells. The same morphological characteristics used to assess the epithelial or mesenchymal nature of untransduced cells were also used to assess the transduced cells (see section 2.1.4).

2.4.3 Protein Analysis

Untransduced, WPI, and *Hoxa7* cells for all cell lines were plated in 10 cm dishes and protein was extracted when the cells reached 80-100% confluence. Cells were prepared for protein extraction by washing twice with PBS. Protein was extracted by adding 500 µl of ProteoJET™ Mammalian Cell Lysis Reagent (Fermentas) + 5 µl Halt Protease Inhibitor Cocktail (Thermo Scientific) to each dish and incubating for 10 minutes at room temperature on a shaker. Lysates were collected in a microcentrifuge tube and sonicated (Sonic Dismembrator Model 300; Fisher Scientific, Ottawa, ON) for 30 seconds. Lysates were clarified by centrifugation at 4°C at 13,000 rpm for 15 minutes. The supernatants containing the protein were transferred to a new microcentrifuge tube and quantified using a commercially available Bio-Rad Protein Assay (Bio-Rad Laboratories, Mississauga, ON). Samples were either used immediately or stored at -80°C for future use.

Forty µg of protein per sample were boiled for 5 minutes in 2X protein loading buffer, centrifuged for 1 minute at 14,000 rpm, and separated on a NuPage 4-12% Bis-Tris polyacrylamide gel (Invitrogen) in a vertical electrophoresis system containing 1X

NuPage MOPS running buffer (20X: 1 M MOPS, 1 M Tris, 69.3 mM SDS, 54.8 mM EDTA; all from BioShop, Burlington, ON). Protein was transferred from the gels to a Hybond-C Extra nitrocellulose membrane (Amersham Biosciences, Buckinghamshire, UK) in 1X NuPage Transfer Buffer (20X: 500 mM bicine, 500 mM Bis-Tris, 20.5 mM EDTA). Membranes were blocked for at least 1 hour in 5% skim milk in 1X Tris-buffered saline Tween-20 (TBST; 150 mM NaCl, 10 mM Tris HCL, 0.1% Tween-20). All membranes were probed with the following primary antibodies: a mouse monoclonal antibody to E-cadherin (1:5000; 610182, BD Biosciences, Mississauga, ON), a rabbit vimentin (1 µg/ml; ab45939, AbCam, Cambridge, MA), and a mouse GAPDH (1:15,000-1:50,000; ab8245, AbCam) as a loading control. Each primary antibody was diluted in 5% skim milk in 1X TBST and incubated with the membranes for 1 hour on a shaker at room temperature. Blots were washed 3 times for 5-10 minutes with 1X TBST, then incubated with the secondary antibody diluted in 5% skim milk in 1X TBST for 1 hour on a shaker at room temperature. The secondary antibody for E-cadherin and GAPDH was a goat anti-mouse horseradish peroxidase-conjugated Immunoglobulin G antibody (IgG HRP; 1:2000; ab6789, AbCam), and a donkey anti-rabbit IgG HRP (1:5000; code 711-035-152, Jackson ImmunoResearch Laboratories Inc., West Grove, PA) for vimentin. Blots were then washed 3 times for 5-10 minutes in 1X TBST. Visualization of protein bands was performed using the ECL Advance™ Western Blotting Detection Kit (Amersham) and the GeneGnome bio imaging system with GeneSnap software (Syngene, Frederick, MD). Densitometry was performed for all blots using the GeneTools program (Syngene). When the blots were to be re-probed with another of the

three primary antibodies, they were first washed 3 times for 10 minutes in 1X TBST to remove the rest of the ECL, then Restore™ Western Blot Stripping Buffer (Thermo Scientific) was applied for 20 minutes. The membranes were then washed again 3 times for 5-10 minutes in 1X TBST before blocking and applying the new primary antibody.

2.4.4 Colony Formation Assays

Growth in soft agar was used as an indicator of transformation in MOSE, MOSE-WPI, and MOSE-*Hoxa7* cells. The human ovarian cancer cell line A2780s was used as a positive control for colony formation. The base agarose mixture was made by melting 2% low melting point (LMP) agarose in the microwave, mixing with an equal volume of 2X α -MEM (MOSE-derived lines) or 2X D-MEM (A2780s) + 10% 3:1 DBS:FBS, and cooling to 37°C in a water bath. 300 μ l of the base agarose mix was added to each well in 24-well plates (1 per cell line; Corning), allowed to solidify for 15 minutes at room temperature, then plates were incubated at 37°C until needed. The top agarose mixture was made by melting 1% LMP agarose, mixing with an equal volume of either 2X α -MEM (MOSE-derived lines) or 2X D-MEM (A2780s) + 10% 3:1 DBS:FBS, and cooling to 37°C until needed.

All cell lines were trypsinized and counted with the Vi-CELL counter (Beckman Coulter), and diluted in 10 ml of the top agarose mixture to yield a concentration of 2000 cells/700 μ l. Cells were serially diluted by one half five times, giving a total of six cell dilutions ranging from 2000 cells/700 μ l to 62.5 cells/700 μ l in the top agarose mixture. 700 μ l of each cell dilution in agarose was plated on 4 wells of the 24-well plates

containing the base agarose layer. The top agarose cell mixture was allowed to set at room temperature for 15 minutes then placed in a 37°C incubator for several weeks. Three independent colony formation assays were performed and colonies were counted at 71-78 days post-plating when colonies were large enough to visualize at 4X magnification with a Wild Heerbrugg light microscope (Leica Microsystems GmbH, Wetzlar, Germany). A colony was defined as any sized cluster of cells that was visible at 4X magnification. The mean percentage of input cells that formed colonies was calculated for each cell density. The mean of these percentages was calculated for each experiment. The overall average percentage of colony forming cells is the average of these percentages from the three independent experiments.

2.5 Immunohistochemistry

Pax8 and TAg protein expression was examined in a panel of 16 tumours from tgMISIIR-TAg mice. Half of the tumours were primary ovarian tumours from control mice (non-ovariectomized). The other half were tumours from mice whose ovaries had been removed (ovariectomized) and therefore arose via MISIIR promoter-driven TAg activation in the uterus and oviduct. Normal mouse oviduct was used as a positive control for Pax8. All tissues were formalin fixed and paraffin embedded. Five µm tissue sections were cut with a microtome, transferred to microscope slides, and allowed to dry for 24 hours. Slides were deparaffinized and hydrated through xylenes and graded alcohol and rinsed in ddH₂O. Antigen retrieval was performed in pH 6 sodium citrate buffer (Antigen Unmasking Solution; Vector Laboratories, Burlingame, CA) for 10-15 minutes in a pressure cooker. Slides were rinsed in tap water and allowed to cool to room temperature.

Endogenous peroxidase activity was blocked by placing all sections in 3% hydrogen peroxide in ddH₂O for 10 minutes. Tissue sections were washed with Stockholm PBS (S-PBS; used for all wash steps) 3 times for 5 minutes. Sections to be stained with a monoclonal mouse TAg antibody at a dilution of 1:100 (sc-147; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were treated as specified in the Vector Mouse on Mouse Kit protocol (Vector). Sections to be stained with a polyclonal rabbit Pax8 primary antibody (10336-1-AP; Protein Tech Group, Chicago, IL) were then covered with Protein Block, Serum-Free (Dako, Carpinteria, CA; all reagents from Dako unless specified otherwise) for 10 minutes to inhibit nonspecific antibody binding. The Pax8 primary antibody was diluted in Antibody Diluent with Background Reducing Components at a concentration of 1:100. Sections were incubated with the primary antibody overnight at room temperature. Each tissue sample also had a no primary antibody control section. After overnight incubation, sections were washed three times for 5 minutes with S-PBS. Pax8 sections were then incubated with the Anti-Rabbit Envision+ System-HRP Labelled Polymer for 20 minutes and washed again three times for 5 minutes with S-PBS. All slides were then immersed in 250 ml of diaminobenzidine (DAB) chromogen solution (Sigma) with 50 µl of 30% hydrogen peroxide for 5 minutes. Sections were immersed in tap water, then counterstained with Harris' hematoxylin (Fisher Scientific) for 1 minute, rinsed in tap water, dipped in 0.2% ammonium water, then rinsed again in tap water. Sections were dehydrated through a series of graded alcohol and xylenes, then mounted with Permount (Fisher Scientific) and coverslips.

Positive pixel analysis of Pax8 and TAg on serial sections of ovariectomized and control tgMISIIR-TAg tumours was performed by Dr. Ken Garson using the Aperio

Scanscope (Aperio Technologies, Inc. Vista, CA). All sections were scanned and visually examined using the ImageScope software (Aperio) for any areas of normal tissue, which were excluded from the analysis. In most cases, only the strong positive pixels for each stain were used for the analysis, as these most clearly reflected the observed nuclear stain (only one case included both "strong positive" and "positive" pixels where this better reflected the nuclear stain). Then the number of positive pixels was divided by the total number of pixels (negative + positive) defined in the tumour area. Finally, the percentage of Pax8-positive pixels was divided by the percentage of TAg-positive pixels, and this value was also expressed as a percentage. These numbers are similar to those generated by simply dividing the number of Pax8 strong positive pixels by the number of TAg strong positive pixels, however, this method helps to account for any slight differences in the total size of the serial sections.

2.6 Statistical Analyses

All statistical analyses were performed using GraphPad Prism statistical software (version 3.02; GraphPad Software, San Diego, CA). For all statistical tests, statistical significance was inferred when $p < 0.05$.

Three independent proliferation assays were done with three replicates per cell line per experiment. Differences between cell lines in the total number of viable cells present at 96 hours post-plating were assessed by one-way ANOVA and Tukey's post test.

Q-PCR analysis of *Hoxa7* expression in untransduced ovarian cancer cells was performed on 3 independent cDNAs, in triplicate on three separate plates. For the Q-PCR

analysis of *Hoxa9*, *Hoxa10*, *Hoxa11*, and *Pax8* in the untransduced cells as well as analysis of *Hoxa7* in the MOSE, MOSE-WPI and MOSE-*Hoxa7* cells, cDNAs from three independent RNA extractions and RT-PCR reactions were pooled and run in triplicate on three separate Q-PCR plates. For *Hoxa7* analysis in the WPI-LV and *Hoxa7*-LV-transduced ovarian cancer cells, cDNAs from one RNA extraction and RT-PCR reaction were run in triplicate on three separate Q-PCR plates. Gene expression is displayed as mean relative expression (after normalization to each cDNA's level of *18S*) $\pm$ standard error of the mean (SEM). The statistical analyses performed for each Q-PCR experiment, wherever possible, included either a one-way ANOVA and Tukey's post test (comparing all groups), or a T test (comparison of one group to a control, usually MOSE). The number of samples per group and the type of test used is indicated in each figure legend.

For Western blots, two (all cell lines but the MOSE) or three (MOSE) independent protein extractions from the untransduced, WPI, and *Hoxa7* cells were used. After normalizing the amount of protein in each sample to GAPDH by densitometry, this normalized value was expressed relative to the protein levels in untransduced MOSE, which were set as 1 for both the E-cadherin and vimentin Western blots. Any changes in the E-cadherin or vimentin protein density among WPI and *Hoxa7* cells compared to untransduced cells were assessed by one-way ANOVA. The same analyses were used for Western blots involving EGF+ and EGF- cells.

Colony forming ability is expressed as the mean percentage of input cells from three independent experiments that form colonies, $\pm$ SEM. For each cell line, this was calculated by determining the percentage of input cells at each density (6 densities per

cell line) that form colonies, then averaging this number, which provided the value for one experiment. Colony forming ability in all cell lines was compared by one-way ANOVA and Tukey's post test.

CHAPTER 3: RESULTS

3.1 Cell line morphology and proliferation rates

The cell lines listed in **Table 1** were used for all experiments. The initial characterization of these cell lines involved taking photomicrographic images of all cell lines in culture to establish morphology. **Figure 7** shows the morphology of the cells. The normal MOSE cell line had a variable phenotype; cells could often be found in cobblestone-like clusters that are typical of epithelial cells, but they also could look elongated and had fibroblast-like projections. The MOSE-RM cells had a very fibroblastic appearance. All three ascites lines (MASC2, MASE2, MASP1) generally looked quite similar. At low densities in particular, they looked very fibroblastic and usually displayed projections or ruffled edges. As they reached confluence, they looked more cobblestone-like. The MISIIR-TAg tumour cell line generally displayed a cobblestone morphology, irrespective of the degree of confluence.

Next, growth curves were conducted to establish and compare the proliferation rates of each cell line. All cell lines were plated at a density of 2×10^4 cells per well and counted every 24 hours over 4 days. **Figure 8** shows that over 96 hours in culture, there is a wide range in growth rates for all the cell lines. At the 96 hour time point, there are significantly more MOSE-RM cells than all other cell lines except the MASC2 cell line. The MOSE-RM cells grow the fastest of all the cell lines with a doubling time of 12.02 hours. There is no significant difference in growth rate between each of the three ascites cell lines, and their doubling times are 12.45 hours for the MASC2 cells, 13.18 hours for

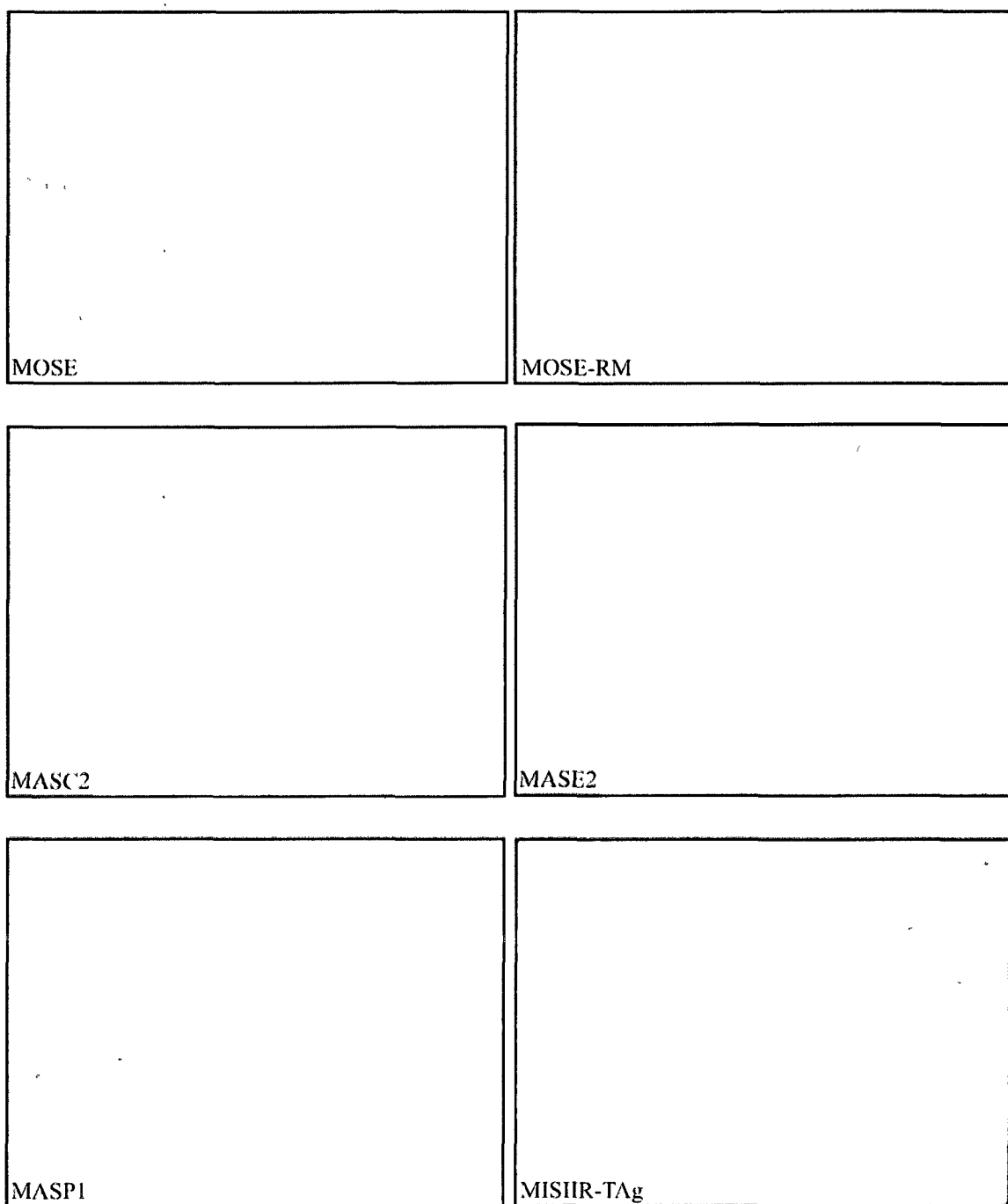


Figure 7: Photomicrographic images of normal MOSE and mouse ovarian cancer cell lines. Live cells were photographed at 200X magnification.

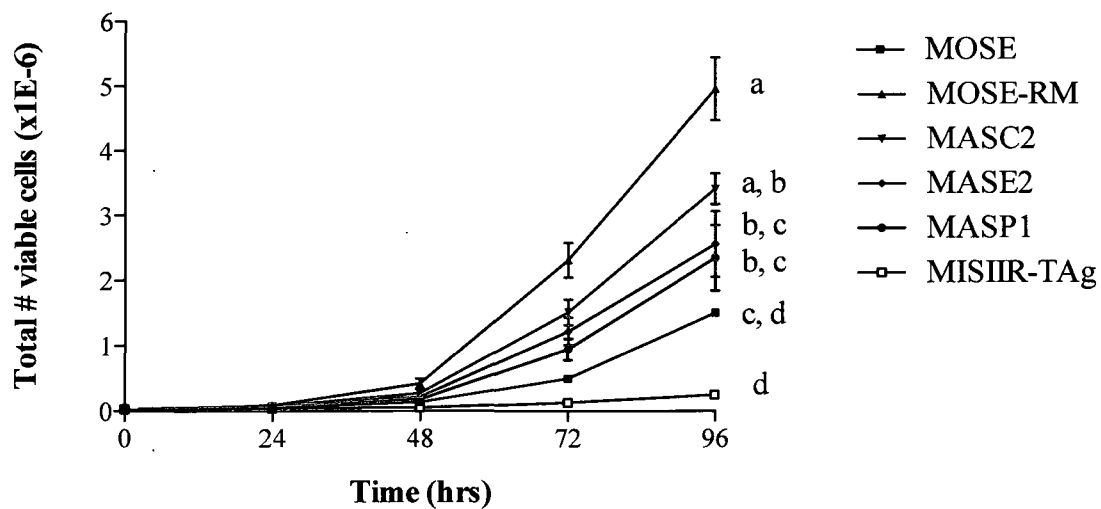


Figure 8: Proliferation rates of normal MOSE and mouse ovarian cancer cell lines.

Cells were plated at a density of 20,000 cells per well in 6-well dishes and counted at 4 time points post-plating. The letters indicated significant differences between groups at 96 hours determined by a one-way ANOVA and Tukey's post test ($p < 0.05$). The doubling times of the cell lines are as follows: MOSE, 14.32 hours; MOSE-RM, 12.02 hours; MASC2, 12.45 hours; MASE2 13.18 hours; MASP1, 13.37 hours; MISIIR-TAg, 25.70 hours.

the MASE2 cells, and 13.37 hours for the MASP1 cells. While the MISIIR-TAg tumour cell line grows the slowest in culture, exhibiting a trend to grow more slowly than the normal MOSE cell line (25.70 hour doubling time for the MISIIR-TAg cells and 14.32 hour doubling time for the MOSE), there is no significant difference between the number of MISIIR-TAg cells and the MOSE cells at 96 hours post-plating.

3.2 Endogenous expression levels of *Hoxa* and *Pax8* genes

Since overexpression of *Hoxa7*, *Hoxa9*, *Hoxa10*, *Hoxa11*, and *Pax8* has been demonstrated in human EOC (Cheng *et al.* 2005; Bowen *et al.* 2007), it was important to investigate the level to which these genes were expressed in our normal MOSE and mouse ovarian cancer cell lines. The baseline expression levels of the *Hoxa* genes in all cell lines examined by Q-PCR are shown in **Figure 9**, and baseline expression of *Pax8* is shown in **Figure 10**. **Figure 9** shows that the MOSE express extremely low levels of endogenous *Hoxa7*, and that all ovarian cancer cell lines express more *Hoxa7* than the MOSE, however there is a great deal of variability in expression levels amongst the cancer cell lines. Comparing each cell line to the MOSE by T test shows that all three ascites cell lines express significantly more *Hoxa7* than the MOSE, whereas the apparent increase is not statistically significant for the MISIIR-TAg or MOSE-RM cell lines. While there is a visible increase in *Hoxa7* message in the MOSE-RM cells compared to the MOSE, there is enough error to render this statistically insignificant at the defined p value of 0.05 ($p = 0.09$). Of note, the cell line that proliferates the fastest (MOSE-RM) expresses the most *Hoxa7*, and the cell line that proliferates the slowest (MISIIR-TAg)

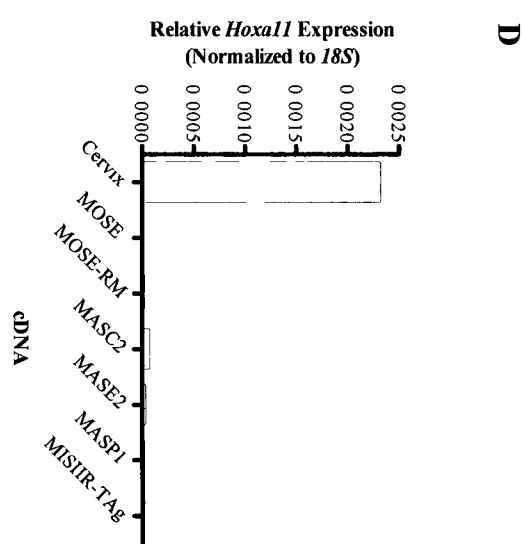
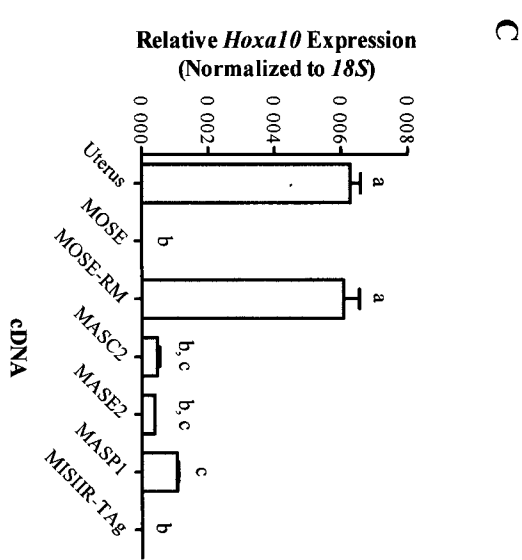
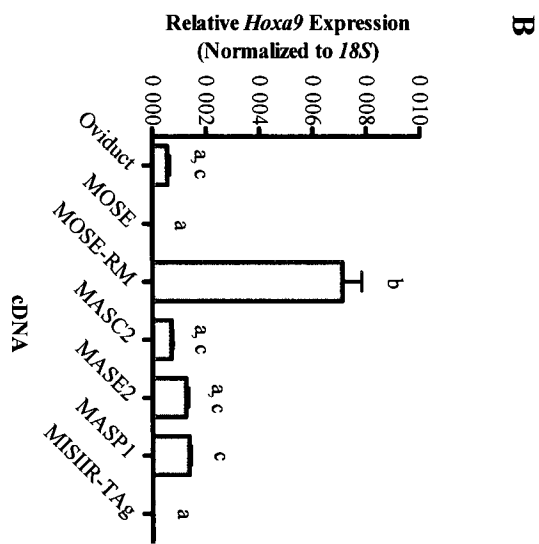
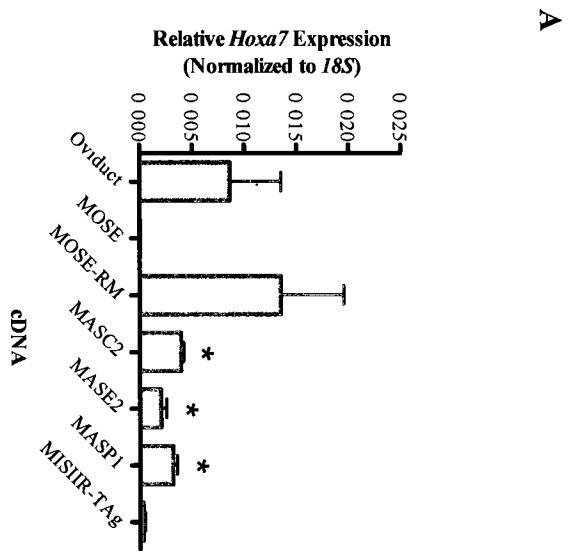


Figure 9: Endogenous *Hoxa7*, *Hoxa9*, *Hoxa10*, and *Hoxa11* mRNA levels in normal MOSE and mouse ovarian cancer cell lines by Q-PCR. (A) Normal MOSE express extremely low levels of endogenous *Hoxa7* mRNA. A statistically significant increase in *Hoxa7* in the cancer cells compared to the MOSE by T test is indicated by an asterisk ($p < 0.05$; $n = 3$). (B-D) Normal MOSE do not express any *Hoxa9*, *Hoxa10*, or *Hoxa11*. The letters indicate statistically significant differences in normalized *Hoxa9* (B), *Hoxa10* (C), or *Hoxa11* (D) expression among cDNA samples when analyzed by one-way ANOVA and Tukey's post test ($p < 0.05$; $n = 3$). A T test cannot be performed comparing each cell line to normal MOSE because MOSE do not express any *Hoxa9*, *Hoxa10*, or *Hoxa11*. Oviduct cDNA was used as a positive control for *Hoxa7* and *Hoxa9* expression, and uterus and cervix were used as positive controls for *Hoxa10* and *Hoxa11*, respectively. Expression of all *Hoxa* genes was normalized to *18S* as an endogenous control.

expresses the least *Hoxa7*. The expression patterns for *Hoxa9* and *Hoxa10* are the same; the MOSE-RM cells express the most *Hoxa9* and *Hoxa10*, the ascites cell lines express intermediate amounts of each transcript, and the MISIIR-TAg cells express the lowest levels of both of these genes. Notably, *Hoxa9* and *Hoxa10* expression is completely absent from normal MOSE, while these genes are still expressed by the majority of the cancer cell lines. Normal MOSE do not express *Hoxa11* either, but mRNA levels of this gene are extremely low to absent from all the ovarian cancer cell lines as well. *Pax8* shows a slightly different pattern of expression among the cell lines compared to the *Hoxa* genes. **Figure 10** shows that normal MOSE express very low levels of *Pax8* mRNA by Q-PCR. However, *Pax8* is undetectable in the MASC2, MASE2 and MISIIR-TAg cell lines. Again, the MOSE-RM cells express the highest levels of *Pax8* in comparison to the other cell lines, but expression of *Pax8* in these cells is also relatively low compared to kidney, the control tissue.

The potential correlation between *Hoxa7* expression and cell growth rate combined with published data indicating that *Hoxa7* may play a proliferative role in granulosa cells in the ovary provided the rationale to enhance *Hoxa7* expression in normal MOSE and ovarian cancer cell lines and assess the consequences on proliferation rate, as well as any impact this might have on cell morphology or expression of epithelial and mesenchymal markers.

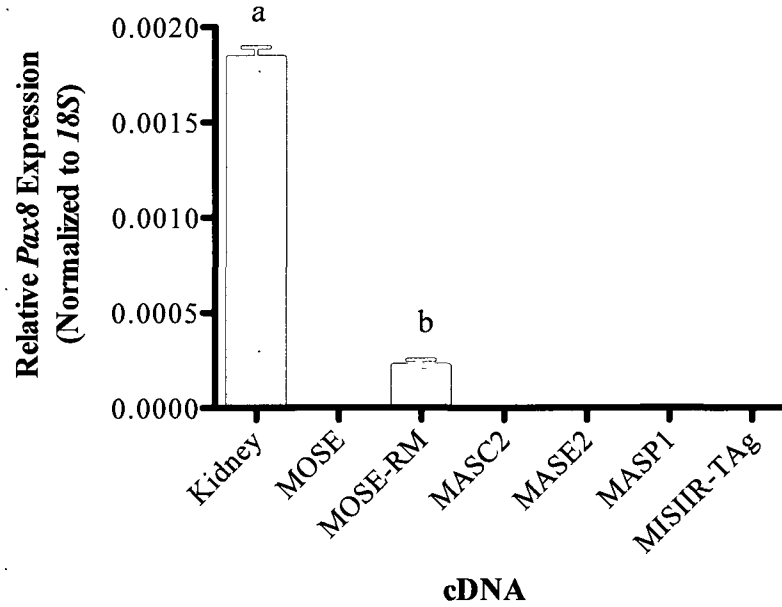


Figure 10: Endogenous *Pax8* mRNA levels in normal MOSE and mouse ovarian cancer cell lines by Q-PCR. Kidney cDNA was used as a positive control for *Pax8* expression. Normal MOSE express extremely low levels of *Pax8*. MASC2, MASE2, and MISIR-Tag cell lines do not express any *Pax8*, and MASP1 cells express very little *Pax8*. MOSE-RM express the most *Pax8* mRNA of all the cell lines. The letters indicate statistically significant differences in normalized *Pax8* expression among cDNA samples when analyzed by one-way ANOVA and Tukey's post test ($p < 0.05$; $n = 3$). The MOSE-RM cells are the only cells that express significantly more *Pax8* than normal MOSE when compared by T test. *Pax8* is normalized to 18S as an endogenous control.

3.3 Effects of *Hoxa7* overexpression

To investigate the consequences of *Hoxa7* overexpression in MOSE and ovarian cancer cells, all cell lines listed in **Table 1** were transduced with the *Hoxa7*-LV or the control WPI-LV (**Figure 6**). Cells were maintained in a mixed population of transduced and untransduced cells until there were sufficient numbers and an appointment could be made for them to be sorted by FACS several weeks after infection. GFP expression was detected by flow cytometry on three separate occasions for the mixed populations of transduced cells (**Figure 11**), excluding the normal MOSE and MISIR-TAg cells, and was used as a measure of the proportion of cells in the mixed population that were successfully infected with the LV. Also, GFP expression serves as an indirect gauge of *Hoxa7* expression in cells transduced with the *Hoxa7*-LV, as GFP is downstream of *Hoxa7* in this construct. **Figure 11A** shows sample histograms for the transduced MOSE-RM cells from each of the three flow cytometry runs, completed at 3, 21, and 56 days post-transduction. As with all the cell lines, untransduced MOSE-RM cells did not fluoresce. However, approximately half of the population from both the WPI-LV and *Hoxa7*-LV-transduced MOSE-RM cells fluoresced, indicating successful viral transduction and subsequent gene expression. Furthermore, the proportion of the population transduced with the *Hoxa7*-LV appeared to increase slightly over time, from 38.2% GFP-positive (GFP+) cells at 3 days post-transduction to 47.47% GFP+ cells at 56 days post-transduction, while the proportion of GFP+ MOSE-RM-WPI cells remained fairly constant over the 56 days in culture after transduction. This suggests that overexpression of *Hoxa7* in the MOSE-RM cells transduced with the *Hoxa7*-LV may

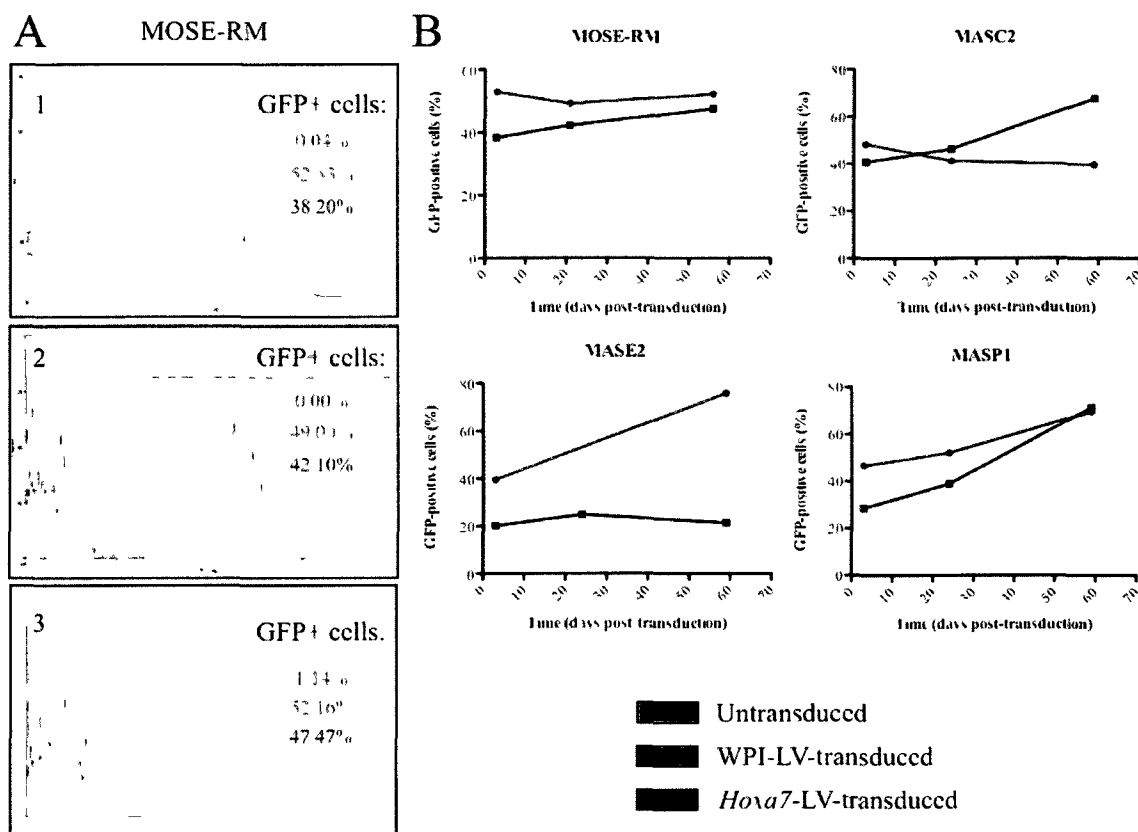


Figure 11: GFP expression detected by flow cytometry in mixed populations of WPI-LV and *Hoxa7*-LV-transduced cells. (A) Representative flow cytometry histograms of untransduced MOSE-RM cells (red) and transduced, unsorted MOSE-RM-WPI (green) and MOSE-RM-*Hoxa7* (blue) cells from three independent runs: 1 (3 days post-transduction), 2 (21 days post-transduction) and 3 (56 days post-transduction). (B) Percentage of GFP-positive cells indicative of transduction with either the WPI-LV or the *Hoxa7*-LV in a mixed population of transduced and untransduced cells, over three independent flow cytometry runs, indicated by each point on the graphs. The runs were performed at 3, 24, and 59 days post-transduction, (except for the MOSE-RM cells, which were run at 3, 21, and 56 days post-transduction).

slightly increase proliferation compared to the vector-transduced cells. **Figure 11B** displays the percentage of GFP+ cells over time in the cell lines for which there were multiple flow cytometry assessments of the mixed populations. The mixed MISIIR-TAg cell population is not represented in this figure because the proportion of GFP+ (and presumably also *Hoxa7*-expressing) cells was found to be quite low at 3.98% when initially assessed by flow cytometry at 22 days after transduction. The GFP+ fraction was sorted by FACS as soon as possible after this assessment in order to avoid potentially losing GFP+ cells from the mixed population. Because it was not known whether this low proportion GFP+ cells in the mixed population of MISIIR-TAg *Hoxa7* cells was due to a lower transduction efficiency of the *Hoxa7*-LV in slow growing cell lines, the MOSE (which also have a relatively low growth rate) were sorted within a few days of transduction before flow cytometry assessment of the mixed population was conducted. Similarly to the trend seen in the MOSE-RM cells, **Figure 11B** indicates that the proportion of GFP+ cells in the *Hoxa7*-LV-transduced MASC2 and MASP1 cells seemed to increase over time, from 40.65% to 67.60% and from 28.47% to 71.04%, respectively. In the case of both of these cell lines, either the proportion of GFP+ cells in the WPI-LV-transduced mixed population did not increase with time (MASC2-WPI), or it did not increase at the same rate as the GFP+ *Hoxa7*-LV-transduced cells (MASP1-WPI). This suggested that the MOSE-RM, MASC2 and MASP1 cells expressing *Hoxa7* proliferated faster than the untransduced cells or those expressing GFP alone. Indeed, the doubling times calculated for these *Hoxa7*-LV-transduced GFP+ cells are slightly shorter than those of the control cells (indicated in **Figure 8**) and of the WPI-LV-transduced cells

(**Table 2**). The proportion of GFP+ cells in each mixed population over the three flow cytometry runs, and the doubling times calculated for the GFP+ cells are displayed in **Table 2**. Given these doubling times, the lengths of time required to see a two-fold difference in the GFP+ *Hoxa7* cells compared to control and WPI cells are indicated in **Table 3**. However, the transduction efficiencies of the WPI-LV and *Hoxa7*-LV in each of the cell lines were never formally established, and not all the cell lines were assessed more than once, so while it may appear that transduction with the *Hoxa7*-LV conferred a growth advantage, these data do not provide sufficient evidence to state that conclusively.

To characterize the potential effects of *Hoxa7* overexpression on the cell lines, LV-transduced, GFP+ cells were sorted by FACS and maintained in culture. All further experiments were performed on these sorted cells. GFP expression was visualized in all cell lines using fluorescent microscopy (**Figure 12**). Increased expression of *Hoxa7* mRNA in the sorted cells was also confirmed by Q-PCR in all cell lines (**Figure 13**). A statistically significant increase in expression of *Hoxa7* of at least 10 fold was displayed in all cell lines transduced with the *Hoxa7*-LV, while there was no significant increase in *Hoxa7* expression in WPI-LV-transduced cells. Western blots probing for Hoxa7 protein in all cell lines were attempted but were unsuccessful due to inconsistent and non-specific antibody binding.

Because there is some evidence in the literature that *Hoxa7* is associated with increased proliferation in granulosa cells in the ovary (Ota *et al.* 2006) and due to our preliminary evidence suggesting increased proliferation in the mixed population of cells transduced with the *Hoxa7*-LV, we assessed the effects of increased *Hoxa7* expression on

Cell Line	GFP+ cells (%)			Doubling time of GFP+ cells (hours)
	3 days post-transduction	24 days post-transduction	59 days post-transduction	
MOSE-RM-WPI*	52.83	49	52.16	12.02
MOSE-RM- <i>Hoxa7</i> *	38.2	42.1	47.47	11.96
MASC2-WPI	48.32	41.09	39.4	12.51
MASC2- <i>Hoxa7</i>	40.65	46.01	67.6	12.32
MASE2-WPI	39.38	N/A	75.71	12.89
MASE2- <i>Hoxa7</i>	20.12	24.75	21.38	13.16
MASP1-WPI	46.56	51.94	69.24	13.19
MASP1- <i>Hoxa7</i>	28.47	38.79	71.04	13.03

Table 2: Percentage of GFP+ cells in a mixed population of transduced and untransduced cells. All cells listed in the table were transduced with either a WPI-LV or *Hoxa7*-LV and assessed for subsequent GFP fluorescence by flow cytometry at three time points following transduction. The proportion of GFP+ cells in the mixed populations of *Hoxa7*-LV-transduced cells generally increase over time. *MOSE-RM cells were assessed at 3, 21, and 56 days post-transduction.

Cell Line	Time (days) after plating at which there are twice as many GFP+ <i>Hoxa7</i> cells as:	
	Untransduced cells	GFP+ WPI cells
MOSE-RM	99.8	99.8
MASC2	49.2	33.8
MASP1	21.4	44.8

Table 3: Time required to achieve a two-fold difference in GFP+ *Hoxa7* cells

compared to untransduced or GFP+ WPI control cells. All values are based on the calculated doubling times of the untransduced cells and of the GFP+ cells from the mixed population of cells transduced with either the *Hoxa7*-LV or the WPI-LV, assuming that all cells are plated at the same density at time 1. The formulas used for these calculations are indicated in section 2.3.4.

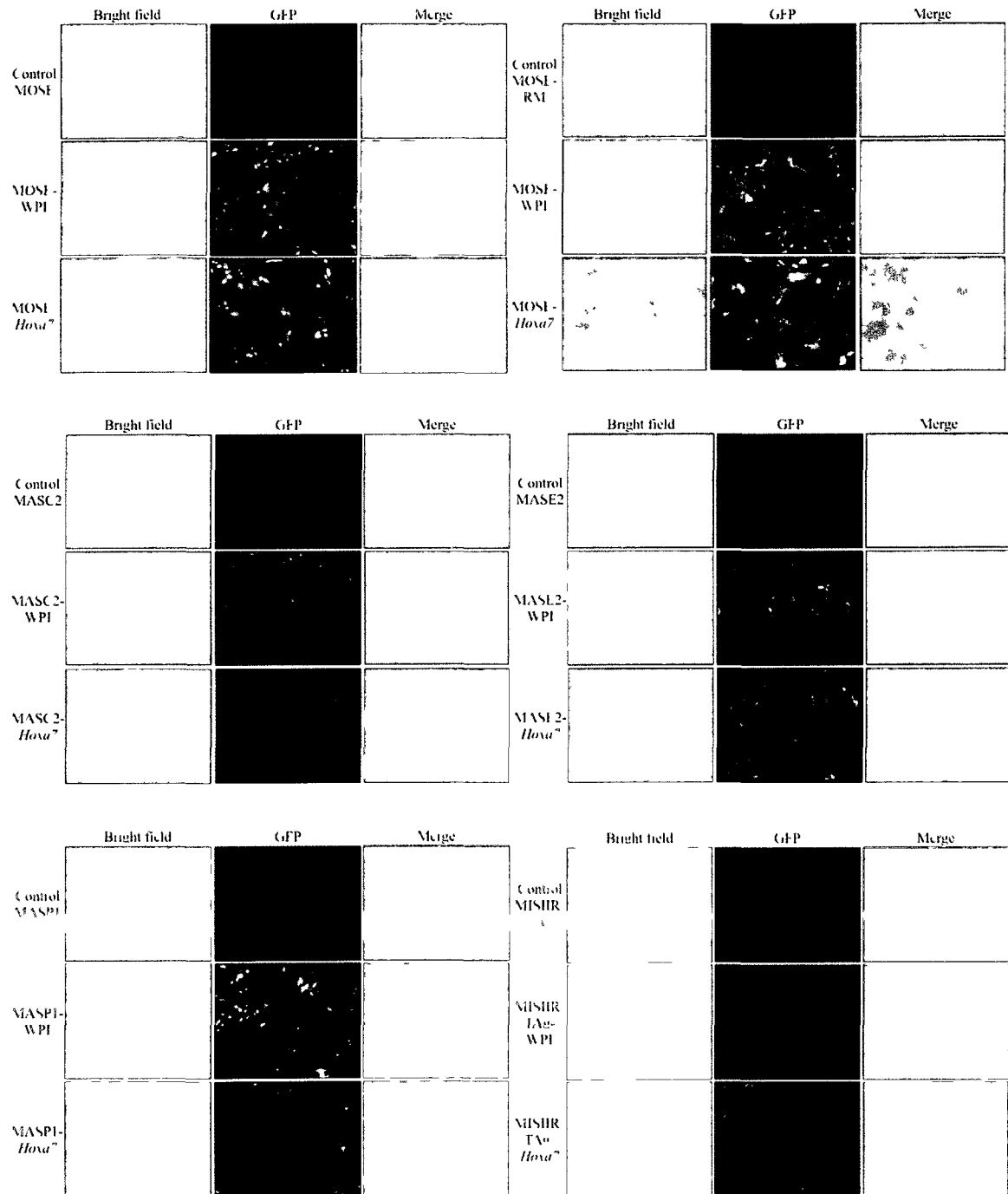


Figure 12: GFP expression in *Hoxa7*-LV and WPI-LV transduced, sorted cells.

Images of all cells were taken with a fluorescent microscope at 200X magnification.

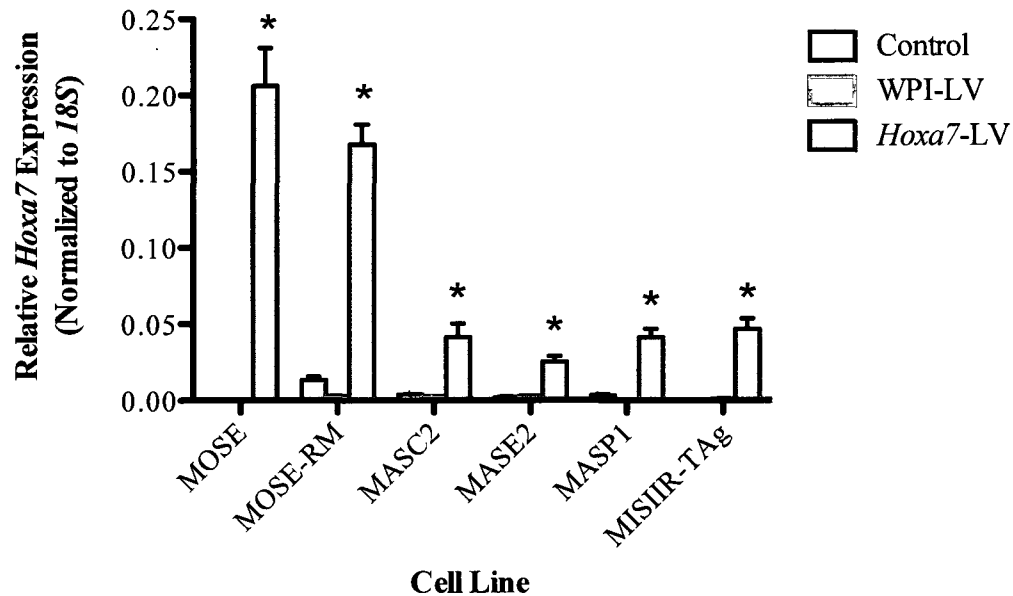


Figure 13: Overexpression of *Hoxa7* in *Hoxa7*-LV-transduced cells. Each cell line was transduced with either the control WPI-LV or the *Hoxa7*-LV, sorted by FACS, and assessed for *Hoxa7* mRNA expression by Q-PCR. *Hoxa7* levels increased by at least 10 fold in the *Hoxa7*-LV-transduced cells relative to the control or vector-transduced cells. The asterisk indicates a statistically significant increase in *Hoxa7* expression in *Hoxa7*-LV-transduced compared to control and vector-transduced cells within each cell line by one-way ANOVA ($p < 0.01$; $n = 3$).

proliferation rates in the transduced, sorted cells. To do this, growth assays of WPI-LV-transduced and *Hoxa7*-LV-transduced cells were conducted in the same manner as for the control cells, and cells were counted every 24 hours for a total of 96 hours. There was no significant difference in the number of cells in each treatment group at any of the four time points for all the cell lines, except for the MOSE-RM cells. From 24 to 72 hours, there were significantly more control MOSE-RM cells than MOSE-RM-WPI or MOSE-RM-*Hoxa7* cells, but by 96 hours after plating there was no significant difference between the number of viable cells in each treatment group, in any cell line (**Figure 14**). Furthermore, though not statistically significant, there was a subtle increase in the number of *Hoxa7* cells present at 96 hours compared to the controls in some of the cell lines, which agreed with the increase over time in the proportion of GFP+ cells within the mixed population of MOSE-RM, MASC2 and MASP1 cells transduced with the *Hoxa7*-LV (**Figure 11**).

There is one paper which showed that transfection of immortalized human OSE cells with *Hoxa7* was sufficient to change the morphology of these cells from that of fibroblastic cells to a more cobblestone-like morphology, which is typical of epithelial cells (Naora *et al.*, 2001a). To examine if overexpression of *Hoxa7* in our cell lines would similarly affect cell morphology, photomicrographic images of all virally transduced cells were taken. As seen in **Figure 15**, there was no change in morphology in any of the cell lines upon transduction with either the WPI-LV or the *Hoxa7*-LV. Despite the lack of alteration in the morphology of *Hoxa7* cells, the next objective was to determine whether overexpression of *Hoxa7* could push MOSE or ovarian cancer cells to become more

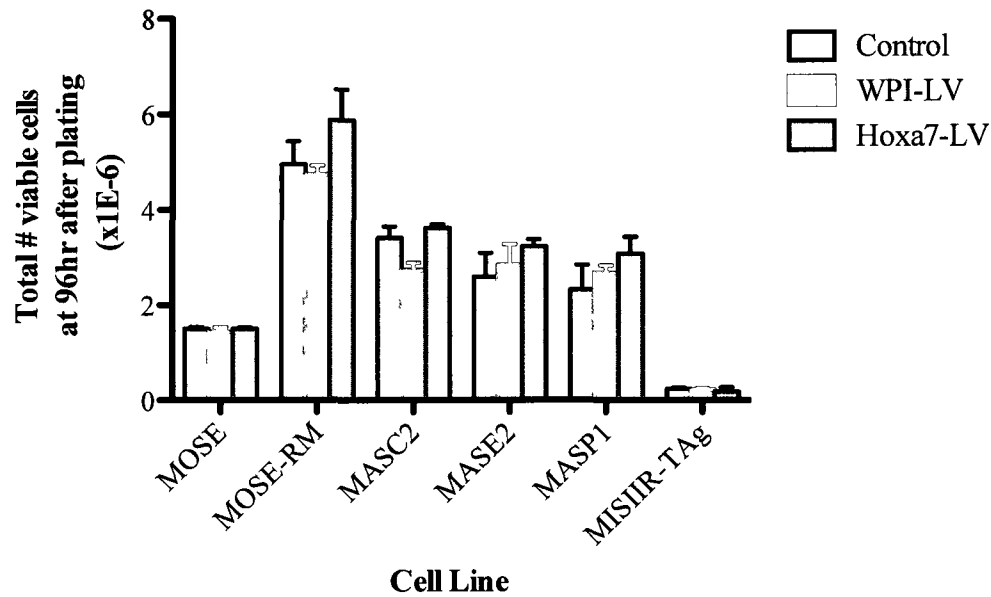


Figure 14: *Hoxa7* overexpression does not affect growth rate in normal MOSE or ovarian cancer cells over 96 hours. All cells were plated at a density of 20,000 cells per well in 6-well dishes and viable cells were counted every 24 hours for 4 days. The total number of viable cells present at 96 hours post-plating for each treatment group of each cell line is displayed. There was no significant difference in the number of viable cells within each treatment group at 96 hours, as determined by one-way ANOVA ($n = 3$).

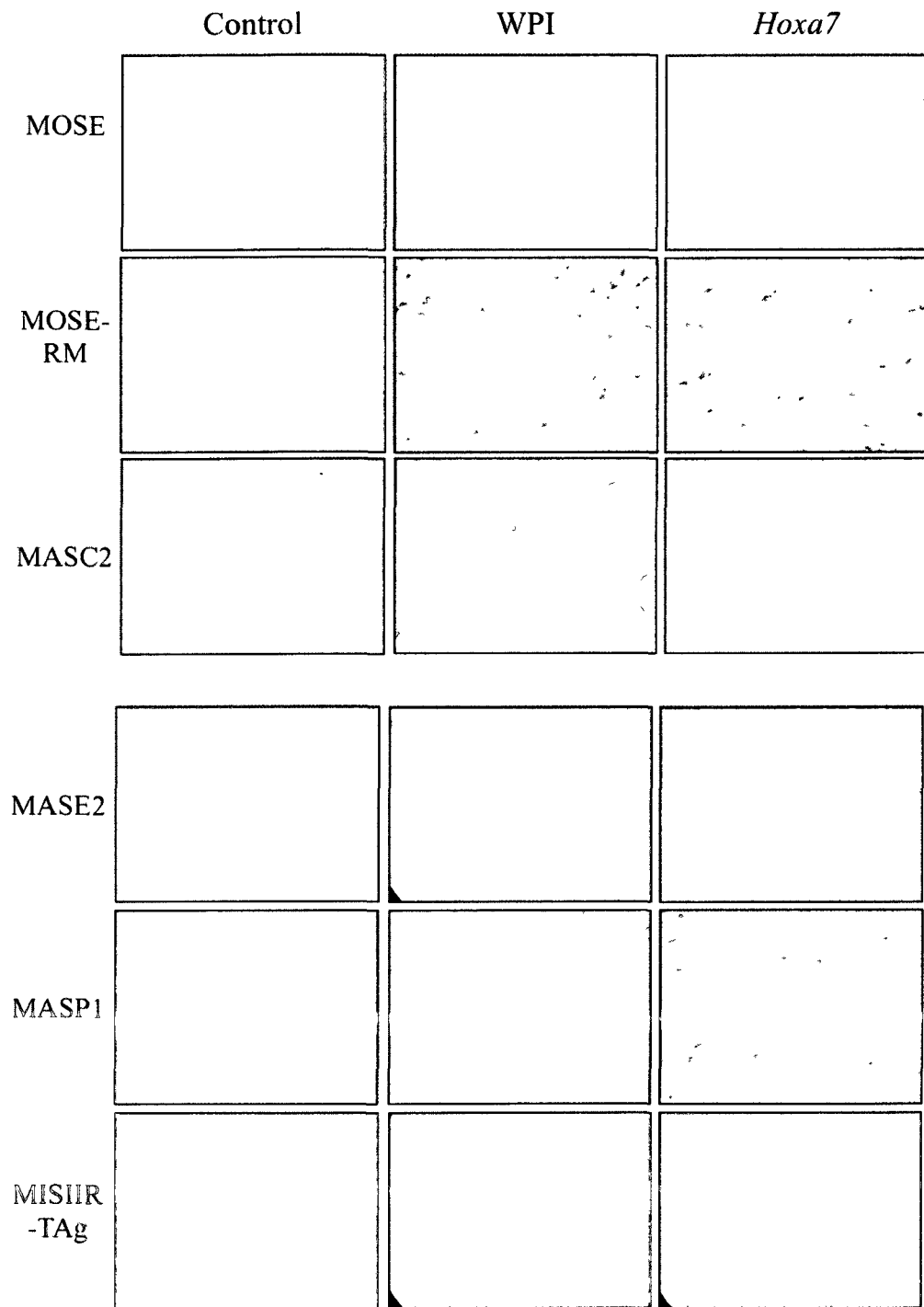


Figure 15: Photomicrographic images of lentivirally-transduced MOSE and ovarian cancer cell lines. Live cells were photographed at 200X magnification. There are no morphological differences in the control, WPI, or *Hoxa7* cells in any of the cell lines.

epithelial-like or less mesenchymal-like, as evidenced by expression of proteins associated with such a change, as the report by Naora *et al.* (2001a) also indicated that transfection of immortalized human OSE cells with *Hoxa7* induced E-cadherin expression and abrogated vimentin expression. Western blots were conducted on MOSE and the mouse ovarian cancer cell lysates probing for E-cadherin, an epithelial marker, and vimentin, a marker of mesenchymal cells. **Figure 16** shows the endogenous expression levels of E-cadherin and vimentin in normal MOSE and each ovarian cancer cell line. It had previously been shown that while E-cadherin expression is low to absent from normal human OSE, it is expressed in all subtypes of EOC (Sundfeldt *et al.*, 1997). Similarly to these E-cadherin expression patterns in the human, the MOSE expressed low levels of E-cadherin, and all the ovarian cancer cell lines expressed more E-cadherin than normal MOSE. The MASE2 cell line expressed the most E-cadherin of all the cancer cell lines. The MOSE also expressed low levels of vimentin. Vimentin expression was variable among the cancer cell lines compared to normal MOSE, but there seemed to be an inverse relationship between E-cadherin and vimentin levels in the cancer cell lines. This was expected as E-cadherin and vimentin are normally markers of two different cell types. The mouse fibroblast cell line NIH 3T3 was used as a positive control for vimentin, and while these cells abundantly expressed vimentin, they did not express any E-cadherin, as expected. Western blots were then performed on control, WPI, and *Hoxa7* cell lysates from all cell lines. Each blot was probed with antibodies against E-cadherin, vimentin, and GAPDH, which served as a loading control (**Figure 17A**). The Western blots were run on protein samples from two or three independent protein extractions for

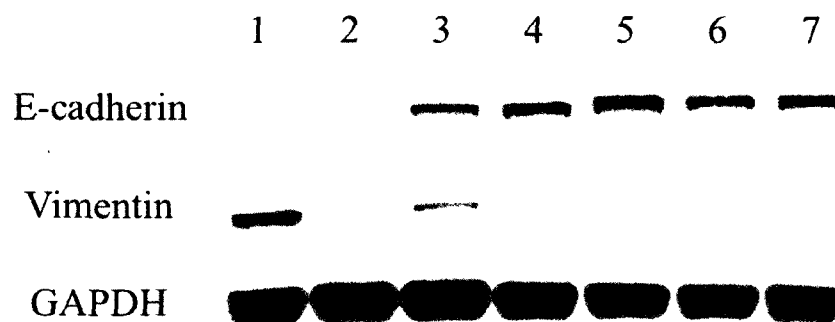


Figure 16: E-cadherin and vimentin protein expression in normal MOSE and

ovarian cancer cell lines. A representative western blot showing endogenous expression of E-cadherin and vimentin in the following cell lines: NIH 3T3 (1; positive control for vimentin), normal MOSE (2), MOSE-RM (3), MASC2 (4), MASE2 (5), MASP1 (6), and MISIIR-TAg (7). The MOSE express low levels of both E-cadherin and vimentin. All cancer cell lines express more E-cadherin than the MOSE, and low levels of vimentin. All blots were probed with GAPDH as a loading control.

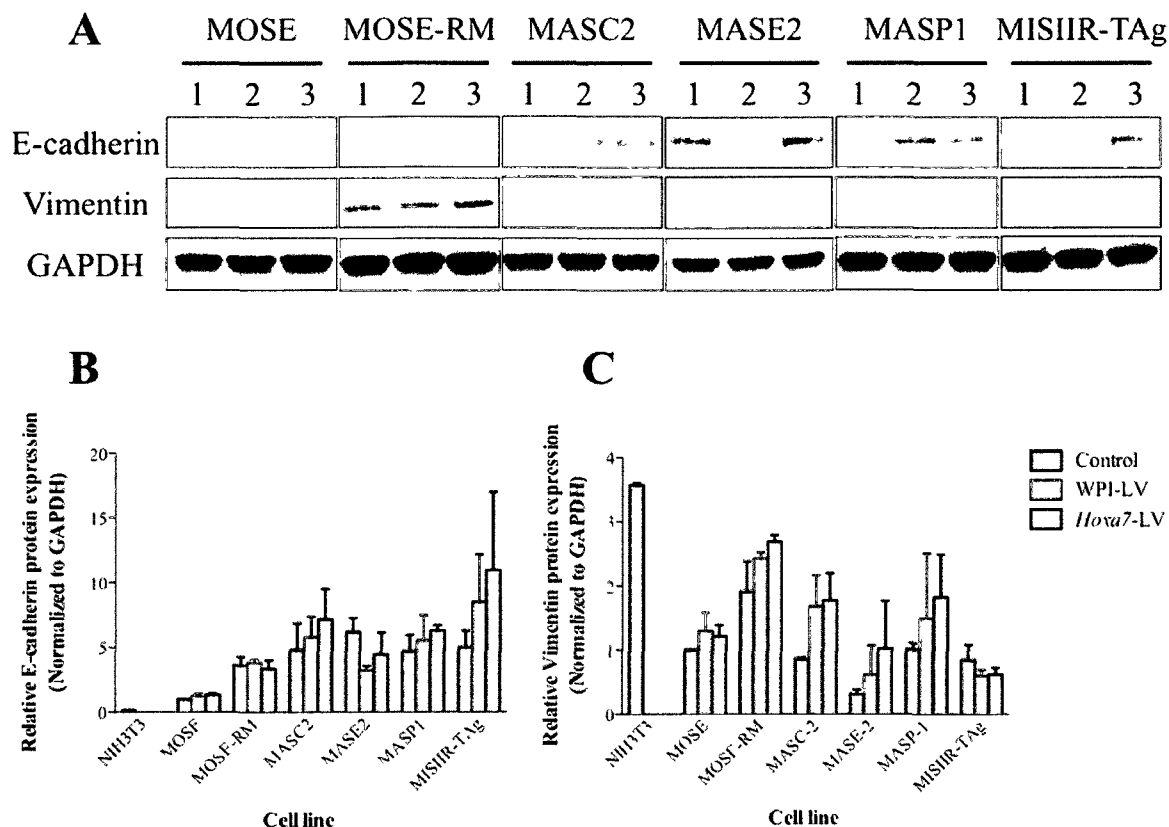


Figure 17: Overexpression of *Hoxa7* does not affect E-cadherin or vimentin protein levels in normal MOSE or ovarian cancer cell lines. (A) Representative western blots of control (1), WPI-LV-transduced (2) and *Hoxa7*-LV-transduced (3) cells probed for E-cadherin, vimentin, and the loading control GAPDH. Quantification of relative E-cadherin (B) and vimentin (C) protein expression in all cells by densitometry analysis of the western blots. There does not appear to be a change in E-cadherin or vimentin expression in any of the cell lines upon *Hoxa7*-LV transduction. Band density for the protein of interest was normalized to that sample's corresponding GAPDH band. Control MOSE was set as 1 and all other samples are displayed relative to control MOSE (n = 3 for the MOSE, n = 2 for all other cell lines).

each cell line and each treatment condition. Densitometry analysis of the western blots was conducted to quantify the relative expression of E-cadherin (**Figure 17B**) and vimentin (**Figure 17C**). Overexpression of *Hoxa7* did not alter E-cadherin or vimentin protein levels within any cell line.

Anchorage independent growth in soft agar is a commonly used indicator of cellular transformation, and since HOXA7 has been found to be expressed in putative preneoplastic lesions (Naora *et al.*, 2001a), colony formation assays were conducted to address the possibility that *Hoxa7* plays a role in ovarian cancer initiation. Control, WPI-LV-transduced and *Hoxa7*-LV-transduced MOSE were suspended at several different densities in soft agar and assessed for colony formation at multiple time points. The human ovarian cancer cell line A2780s was used as a positive control for colony formation. Normal control MOSE do not readily form colonies in soft agar at any density (the highest being 2000 cells per well in 24-well dishes), and there was no increase in colony forming ability in *Hoxa7*-transfected MOSE (**Figure 18**). Taken together, these data suggest that *Hoxa7* overexpression alone is neither sufficient to drive the epithelial differentiation of normal MOSE or mouse ovarian cancer cells, nor to transform normal MOSE.

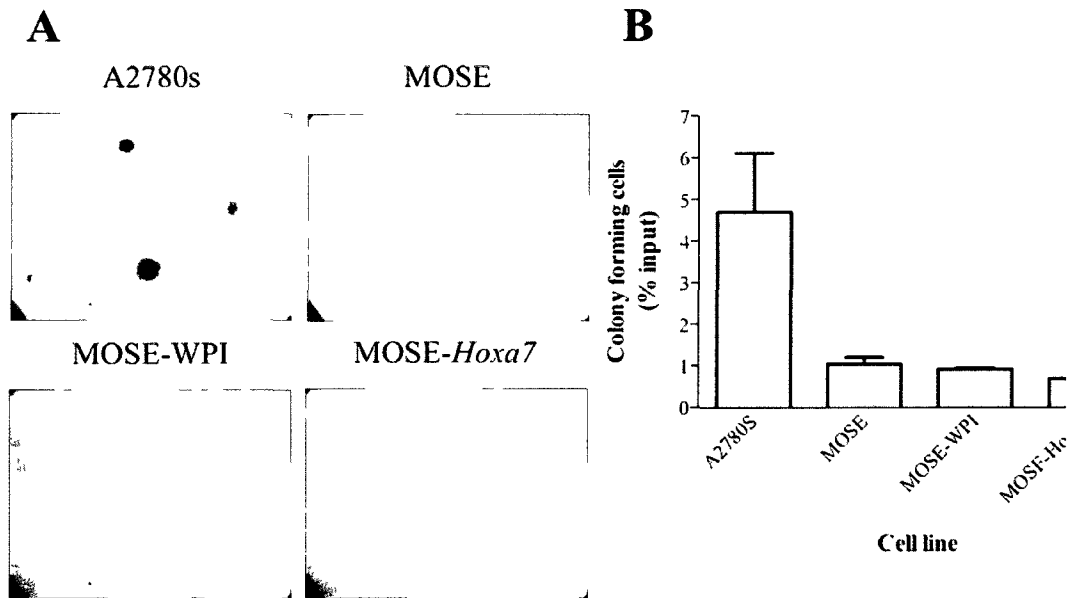


Figure 18: Overexpression of *Hoxa7* does not enhance colony formation in normal MOSE. (A) Photomicrographic images of soft agar plates showing large colonies only in the A2780s positive control wells, taken 51 days after plating at 10X magnification. Normal MOSE form very few, extremely small colonies, and this is not enhanced by transduction with either the control WPI-LV or the *Hoxa7*-LV. (B) Quantification of colony formation expressed as the percentage of input cells that form colonies (A2780s: n = 5; MOSE: n = 3; MOSE-WPI and MOSE-*Hoxa7*: n = 2).

3.4 *Hoxa7* expression in tumours and ascites

To assess the possibility that the cell lines do not reflect the gene expression or cell behaviour of ovarian cancer *in vivo*, the *Hoxa7* expression profiles of the cultured cell lines were compared to those of their corresponding primary tumours from mouse models of EOC by Q-PCR. Interestingly, it was observed that all three cultured ascites lines expressed at least ten fold more *Hoxa7* mRNA than tumours from the models from which those cell lines were derived (**Figure 19**). The MISIIR-TAg cell line, which is derived from a tgMISIIR-TAg mouse ovarian tumour, expressed the same amount of *Hoxa7* as a tgMISIIR-TAg tumour sample. To control for potential inclusion of normal tissue in the tumour and ascites samples, *Tag* mRNA levels were measured in all samples (**Figure 20A**). The primary tumours generally expressed as much, if not more, *Tag* than the cell lines. Even after controlling for *Tag*, all ascites cell lines still expressed more *Hoxa7* than the primary tumours, though the magnitude of this difference ranged quite a bit: 3.5 fold (tgGAG-LS-TAg E2-treated), 17.5 fold (tgCAG-LS-TAg P4-treated), and 86 fold (tgCAG-LS-TAg control) (**Figure 20B**). The ascites cell lines and the primary tumours were also assessed for expression of *Hoxa9* and *Hoxa10*. **Figure 21A** shows that there seemed to be a tendency for the MASE2 and MASP1 ascites cell lines to express more *Hoxa9* than their corresponding primary tumours and for the MASC2 cell line to express less *Hoxa9* than the tgCAG-LS-TAg control tumours. However, **Figure 21B** shows that this expression pattern did not remain after normalizing for TAg; then there was a trend for all three ascites cell lines to express more *Hoxa9* relative to TAg than the primary tumours. In contrast to the expression patterns of *Hoxa7* and *Hoxa9*, the primary

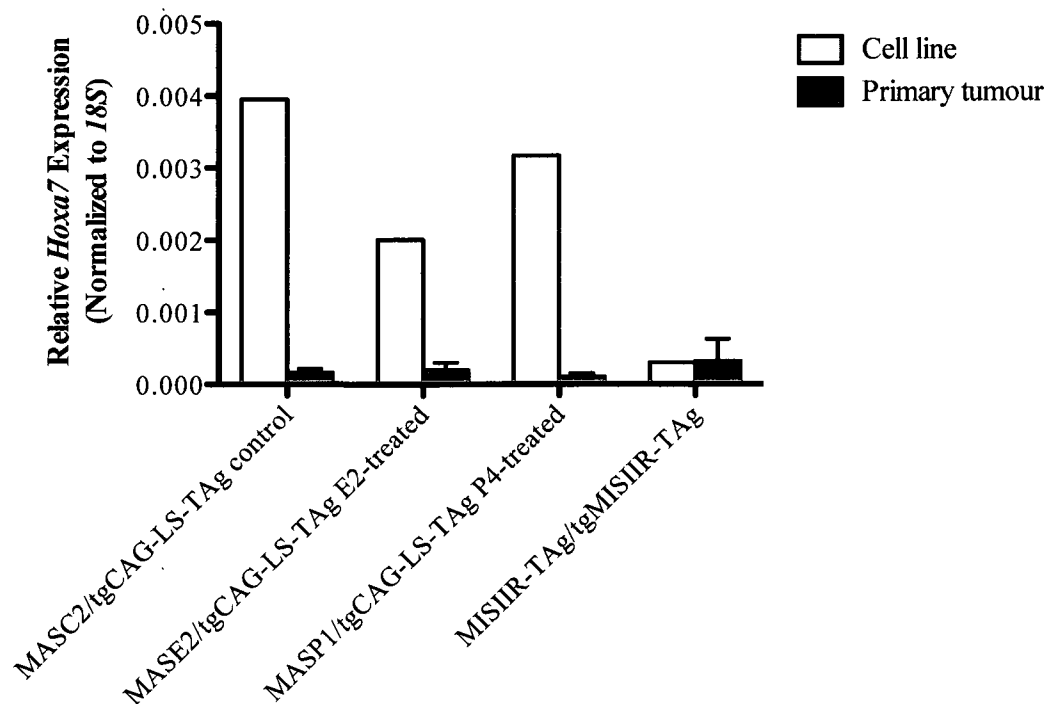


Figure 19: Cultured ascites cell lines express more *Hoxa7* than primary tumours from the same mouse model of EOC. *Hoxa7* mRNA expression (relative to *18S* as an endogenous control) was determined by Q-PCR for cultured, murine ovarian cancer cell lines and primary tumour samples from the same mouse models from which the cell lines were derived. All cultured ascites cell lines from the tgCAG-LS-TAg mouse model of EOC expressed more *Hoxa7* than their corresponding primary tumours; the fold difference in *Hoxa7* in the ascites cell lines compared to the tumours was as follows: 20 fold (MASC2:tgCAG-LS-TAg control), 10 fold (MASE2:tgCAG-LS-TAg E2-treated) and 30 fold (MASP1:tgCAG-LS-TAg P4-treated). The MISIIR-TAg tumours expressed the same amount of *Hoxa7* as the MISIIR-TAg cell line (1 cell line and 3 tumours per group).

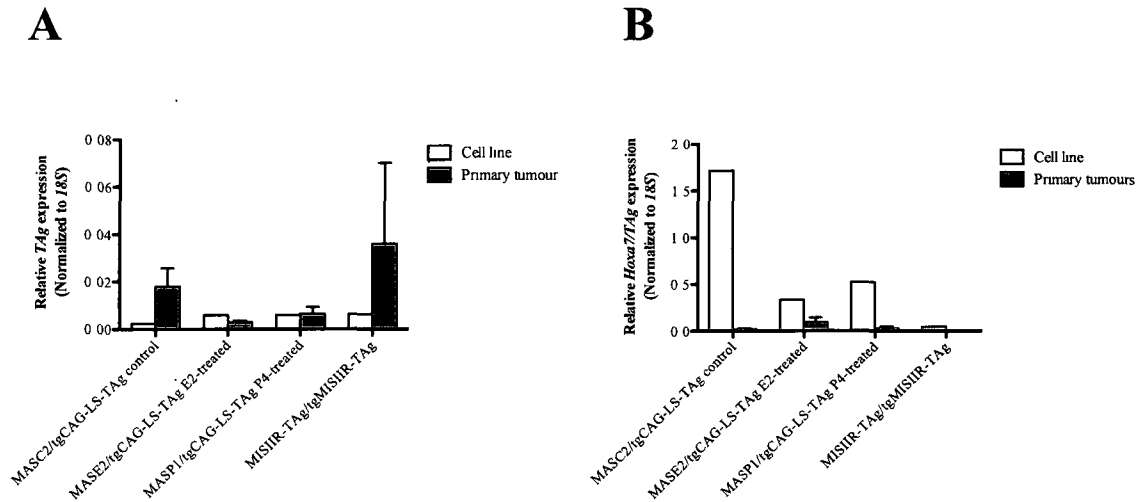


Figure 20: TAg normalization does not change the pattern of *Hoxa7* expression in cell lines compared to primary tumours. (A) TAg expression in mouse ovarian cancer cell lines and primary tumours from mouse models of EOC, relative to *18S* as an endogenous control. tgCAG-LS-TAg control, P4-treated tgCAG-LS-TAg, and MISIIR-TAg tumours expressed 9X, 1.2X, and 6X more TAg than their corresponding cell lines, respectively. MASE2 cells expressed 2X more TAg than tgCAG-LS-TAg E2-treated tumours. (B) Relative *Hoxa7*/TAg expression in mouse ovarian cancer cell lines and primary tumours from mouse models of EOC. Even after adjusting for TAg expression in all samples, ascites cell lines still expressed more *Hoxa7* than their corresponding primary tumours; the fold difference in *Hoxa7* in the ascites cell lines compared to the tumours is as follows: 86 fold (MASC2:tgCAG-LS-TAg control), 3.5 fold (MASE2:tgCAG-LS-TAg E2-treated) and 17.5 fold (MASP1:tgCAG-LS-TAg P4-treated). *Hoxa7*/TAg was 9.4X higher in the MISIIR-TAg cell line than in the MISIIR-TAg tumours (1 cell line and 3 tumours per group).

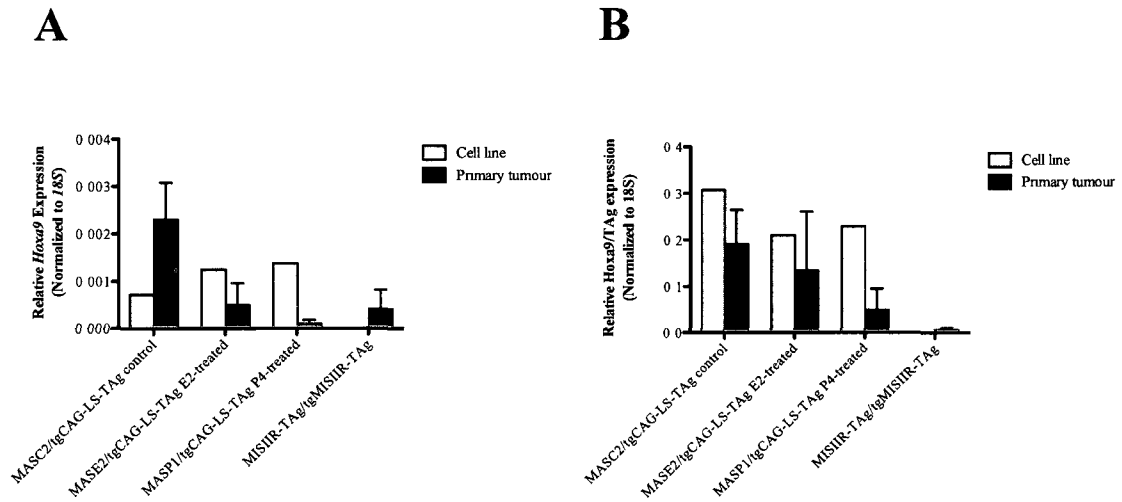


Figure 21: *Hoxa9* expression in cultured cell lines and primary tumours from mouse models of EOC. (A) *Hoxa9* expression relative to 18S was 2.5X higher in MASE2 cells than in the tgCAG-LS-TAg E2-treated tumours, and 16.4X higher in MASP1 cells than in tgCAG-LS-TAg P4-treated tumours; however, it was 2.5X higher in control tgCAG-LS-TAg tumours than in MASC2 cells and 72X higher in MISIIR-TAg tumours than in the MISIIR-TAg cell line. (B) After *Tag* normalization, there was a trend for relative *Hoxa9* expression to be higher in all the ascites cell lines than in the tgCAG-LS-TAg primary tumours (1.6X higher in MASC2 and MASE2 cells than in control and E2-treated tumours, and 4.9X higher in MASP1 cells than in P4-treated tumours). *Hoxa9/Tag* expression was 4X higher in the MISIIR-TAg tumours than in the MISIIR-TAg cell line (1 cell line and 3 tumours per group).

tumours appeared to express as much or more *Hoxa10* than the ascites cell lines, and this trend was maintained very faithfully after TAg normalization (**Figure 22**).

The observation that the ascites cell lines consistently expressed more *Hoxa7* than the primary tumours was particularly interesting and lead us to investigate the possibilities that either this observed difference was comparable to a difference in gene expression between ascites and tumours *in vivo*, or it was a consequence of culturing the ascites cells. Though we initially compared ascites cell lines and primary tumours from the same mouse models of EOC, they were not necessarily from the same animal. To ascertain whether *Hoxa7* expression truly differs between ascites and tumours, we examined *Hoxa7* transcript levels in paired samples of tumours and uncultured ascites cells taken from SCID mice that had been xenografted with MASC1 (a distinct cell line from MASC2, which was used for all other experiments, though it is also an ascites cell line from a tgCAG-LS-TAg control mouse) and MASE2 cells. This revealed that, contrary to what was seen previously, the ascites cells expressed less *Hoxa7* than their paired tumour counterparts (**Figure 23**). In addition, this pattern was maintained when TAg normalization was performed (**Figure 24**). Some of the SCID mice subcutaneously received hormone pellets (that released E2 for 60 days) at the time they were xenografted with MASC1 cells, and the mean *Hoxa7* levels decreased by more than half in both the ascites and tumours from these animals compared to animals that received tumour cells but not E2. This indicated a potential suppressive effect of estrogen on *Hoxa7* expression.

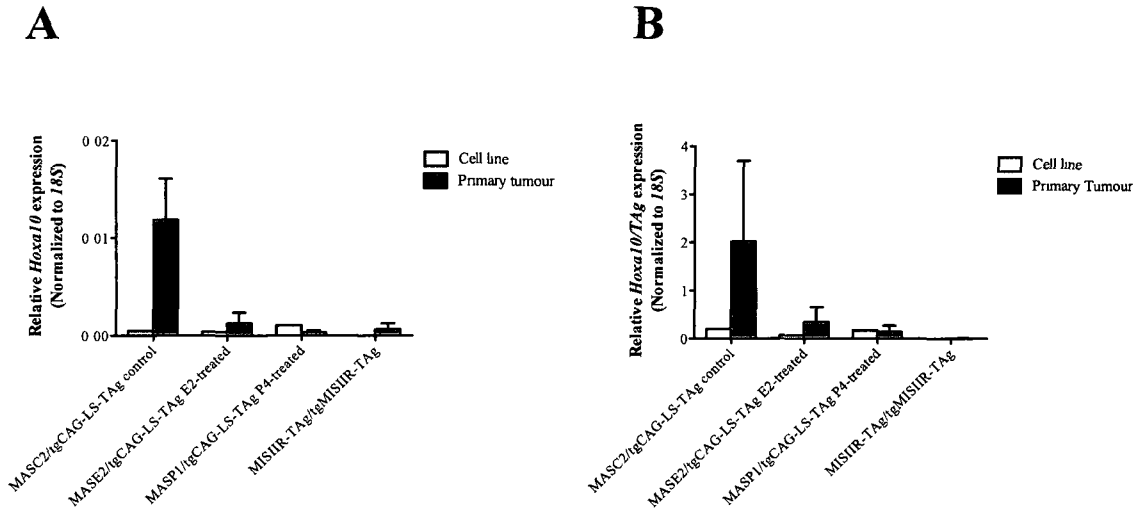


Figure 22: *Hoxa10* expression in cultured cell lines and primary tumours from

mouse models of EOC. (A) *Hoxa10* expression relative to 18S was generally higher in

the primary tumours than in the cell lines: 25X greater in control tgCAG-LS-TAg

tumours than in MASC2 cells, 2.5X greater in the tgCAG-LS-TAg E2-treated tumours

compared to the MASE2 cells, and 222X higher in MISIIR-TAg primary tumours than in

the MISIIR-TAg cell line. However, it was 3.4X higher in the MASP1 cells than in

tgCAG-LS-TAg P4-treated tumours. (B) After *Tag* normalization, relative *Hoxa10*

expression still appeared to be higher in the primary tumours than in the cell lines, though

the magnitude of this difference was often reduced. *Hoxa10/Tag* expression was 9.8X

higher in control tgCAG-LS-TAg tumours than in MASC2 cells, 5.2X higher in the

tgCAG-LS-TAg E2-treater tumours than in the MASE2 cells, and 11X higher in MISIIR-

Tag primary tumours than in the MISIIR-TAg cell line. However, it was 1.2X higher in

the MASP1 cells than in tgCAG-LS-TAg P4-treated tumours (1 cell line and 3 tumours

per group).

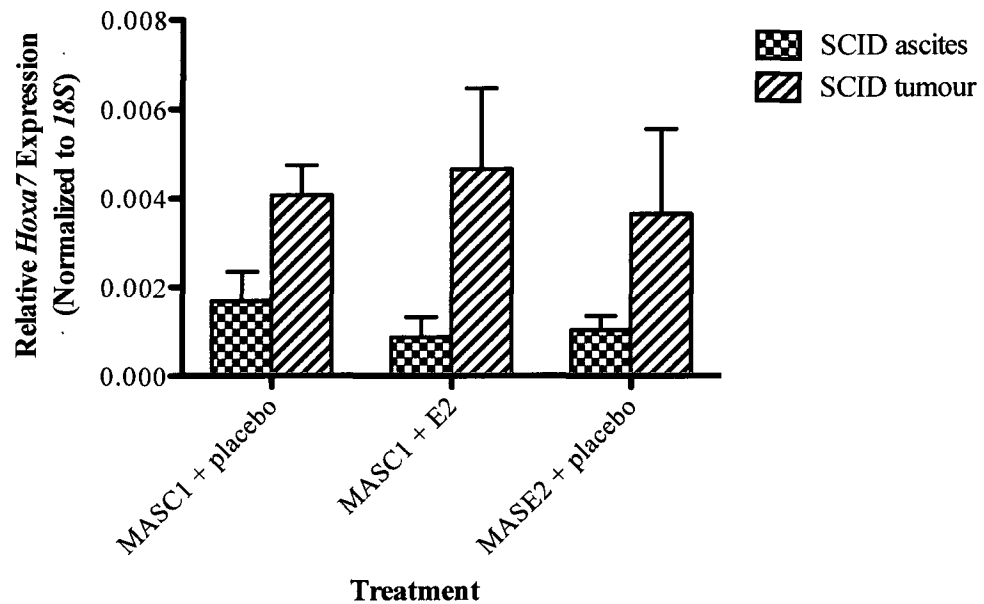


Figure 23: Uncultured SCID mouse ascites express less *Hoxa7* than their paired

SCID mouse tumours derived from xenograft studies. Tumours and ascites taken from the same SCID animal after IP injection with either MASC1 or MASE2 cells, and treatment with either E2 or placebo, were assessed by Q-PCR for expression of *Hoxa7*. Regardless of the cell line or treatment, the ascites samples express less than half as much *Hoxa7* as their paired tumour (n = 2 per group).

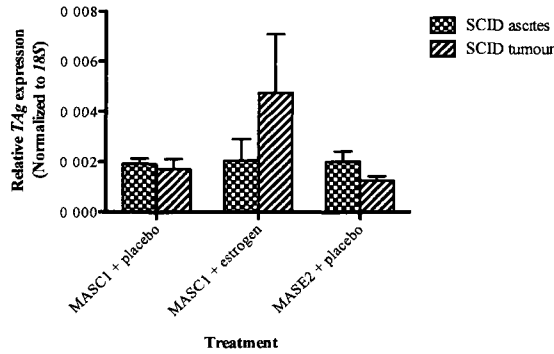
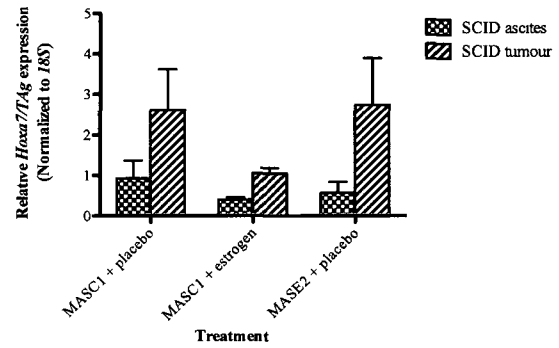
A**B**

Figure 24: *Tag* normalization does not change the pattern of *Hoxa7* expression

SCID mouse ascites compared to their paired SCID mouse tumours derived from

xenograft studies. (A) *Tag* expression in paired SCID mouse ascites and tumours from

xenograft studies, relative to *18S* as an endogenous control. Ascites and tumours from the

MASC1 + placebo group express the same amount of *Tag*. MASC1 + E2 tumours

express 2.3X more *Tag* than ascites, and MASE2 + placebo ascites express 1.6X more

***Tag* than tumours. (B) Relative *Hoxa7/Tag* expression in paired SCID mouse ascites and**

tumours from xenograft studies. Even after adjusting for *Tag* expression in all samples,

SCID ascites still expressed less *Hoxa7* than their paired SCID tumours; the fold increase

in *Hoxa7* in the SCID tumours compared to the SCID ascites is as follows: 2.8 fold

(MASC1 + placebo), 2.6 fold (MASC1 + E2) and 4.8 fold (MASE2 + placebo). (n = 2

per group). Furthermore, both the ascites and tumours from MASC1 + E2 mice expressed

less *Hoxa7* than those from MASC1 + placebo mice (2.3X less in the E2 ascites and 2.5X

less in the E2 tumours compared to the placebo ascites and tumours).

3.5 Effect of *in vitro* culture conditions on *Hoxa7* expression

There is some evidence that EGF can induce the expression of *Hoxa7* in cells in culture (La Celle and Polakowska, 2001), and since EGF is a factor that is added to the cell culture media used for all experiments, the influence of EGF on *Hoxa7* expression on cells was investigated in two experiments. In the first experiment, all cell lines were split into EGF+ and EGF- media and maintained in culture for 3-4 passages. Cells (now referred to as EGF+ and EGF- cells) were subsequently assessed for *Hoxa7* by Q-PCR. There was no influence of maintenance with or without EGF on *Hoxa7* expression in any of the cell lines (**Figure 25A**). In the second experiment that included a third group (EGF-/ +), in which EGF was added back to EGF- cells for 48 hours, there was still no change in *Hoxa7* in either the cells that were maintained without EGF and in those that had EGF- added back after several passages of EGF starvation (**Figure 25B**). However, assessment of the cells from the first experiment revealed that the morphology seemed to be somewhat altered in the MOSE, MASC2 and MASP1 lines that were cultured in the absence of EGF; irrespective of the degree of confluence, EGF- cells appeared to be flatter and had less distinct cell boundaries than EGF+ cells (**Figure 26A**). There is also a trend for these EGF- cells to express more E-cadherin than those cultured with EGF (**Figure 26B,C**).

It is possible that when the ascites cells were removed from tumour-bearing mice and placed in culture, the cell lines gained *Hoxa7* expression as a result of being in adherent culture conditions; these conditions are unlike the *in vivo* environment in which

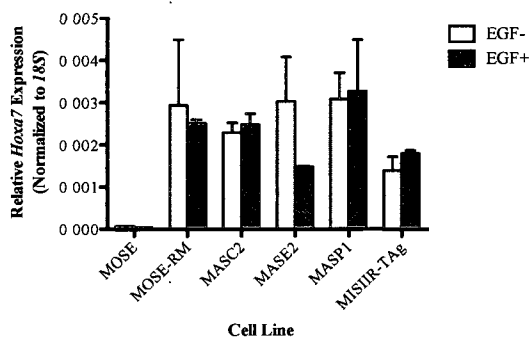
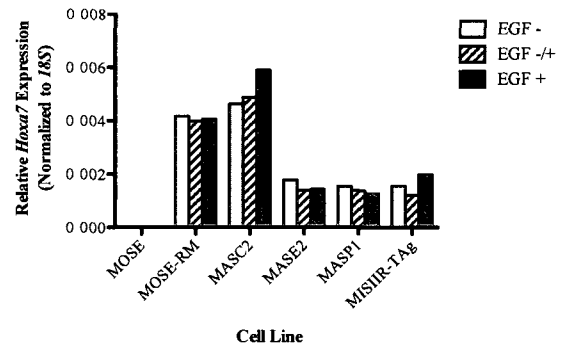
A**B**

Figure 25: EGF has no effect on *Hoxa7* expression *in vitro* in normal MOSE and mouse ovarian cancer cell lines. (A) Cells maintained for 3-4 passages in the media with or without EGF express the same levels of *Hoxa7* normalized to *18S* as an endogenous control (n = 2). (B) Cells maintained without EGF, with EGF added back for 48 hours, and those that always were exposed to EGF have the same expression of *Hoxa7* normalized to *18S* as an endogenous control (n = 1).

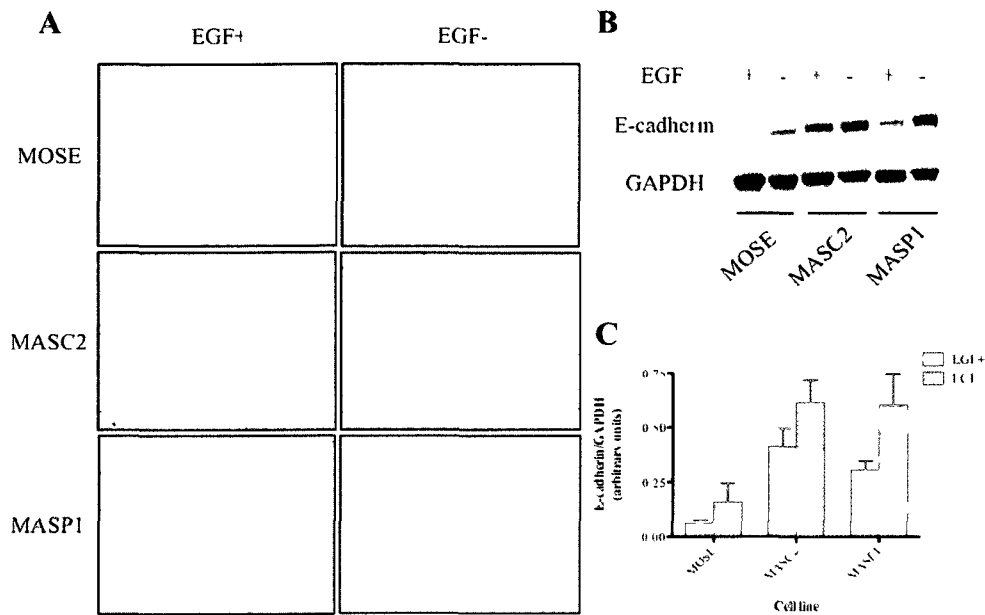


Figure 26: EGF affects the morphology and E-cadherin expression of normal MOSE and mouse ovarian cancer cells *in vitro*. (A) Representative photomicrographic images at 200X magnification of cells that have been maintained in media with or without EGF. EGF- cells began to spread out and became flatter looking with less distinct cell boundaries than EGF+ cells. With respect to the MASC2 cells in these images in particular, there were fewer EGF- cells than EGF+ cells, yet there were no empty spaces between EGF- cells, unlike the EGF+ cells. (B) Representative western blot showing increased E-cadherin expression in EGF- cells compared to EGF+ cells. (C) Relative quantification of E-cadherin expression normalized to GAPDH as a loading control. While not statistically significant by T test, there is a trend for EGF- cells to express more E-cadherin than the EGF+ cells in all three cell lines examined (n = 3; MOSE: p = 0.35, MASC2: p = 0.21, MASP1: p = 0.12).

ascites cells usually exist. To address this possibility, MASC2, MASE2 and MASP1 cells were cultured in plastic bacterial plates that had been coated with agarose. These cells were unable to plate down, but survived and grew in suspension, as ascites cells would have to do within the peritoneal space. Viability of these cells was always confirmed with the Vi-CELL counter and was usually over 90% and always over 80%. The ascites cells cultured in these conditions formed spherical clusters of cells, images of which are shown in **Figure 27A**. *Hoxa7* levels of the suspension ascites cells were compared to the attached ascites cells by Q-PCR, and despite the differing culture conditions, the suspension cells expressed the same amount of *Hoxa7* as the attached cells (**Figure 27B**). These data suggest that though there may be an *in vitro* culture effect that increases *Hoxa7* expression in the ascites cell lines compared to primary tumours or uncultured ascites, it cannot be explained by the presence or absence of EGF alone, or by attachment culture conditions.

3.6 *Pax8* expression in tgMISIIR-TAg and tgLS-CAG-TAg tumours

Since *Pax8* is related to the *Hox* genes with respect to its normal function and its expression in EOC, we wanted to investigate its expression in our mouse models of EOC. First, we confirmed by IHC that normal MOSE do not express Pax8 protein, however the epithelial cells of the oviduct and the uterus do (**Figure 28**). However, as seen in **Figure 10**, all the mouse ovarian cancer cell lines (with the exception of the MOSE-RM) expressed little to no *Pax8* mRNA. We considered that, similarly to the situation with *Hoxa7*, the expression of *Pax8* in ovarian cancer cells *in vitro* may not be consistent with

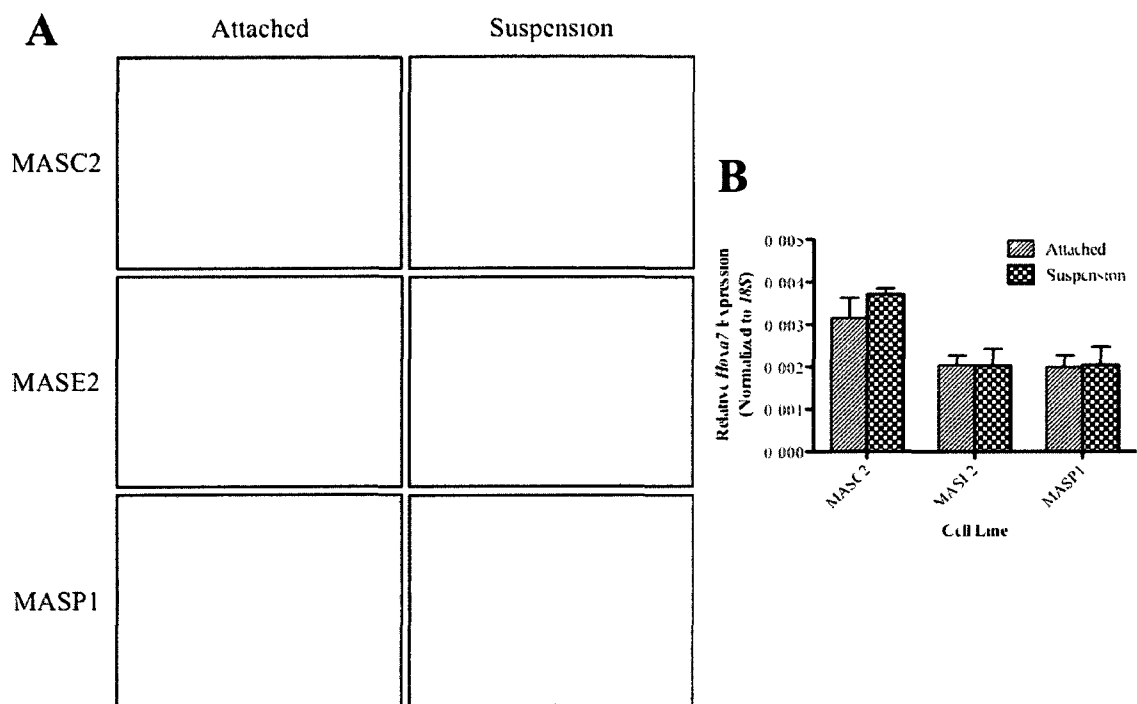


Figure 27: Suspension culture does not affect *Hoxa7* expression in mouse ascites cell lines. (A) Photomicrographic images at 200X magnification comparing ascites cell lines grown in attachment or suspension culture conditions. 5×10^5 MASC2 and MASP1 cells, and 1×10^6 MASE2 cells were added to 10 ml of media on agarose-plated dishes and images were taken 3 days later. (B) Ascites cell lines in both the attachment and suspension culture conditions express the same amount of *Hoxa7* mRNA relative to *18S* as an endogenous control ($n = 3$).

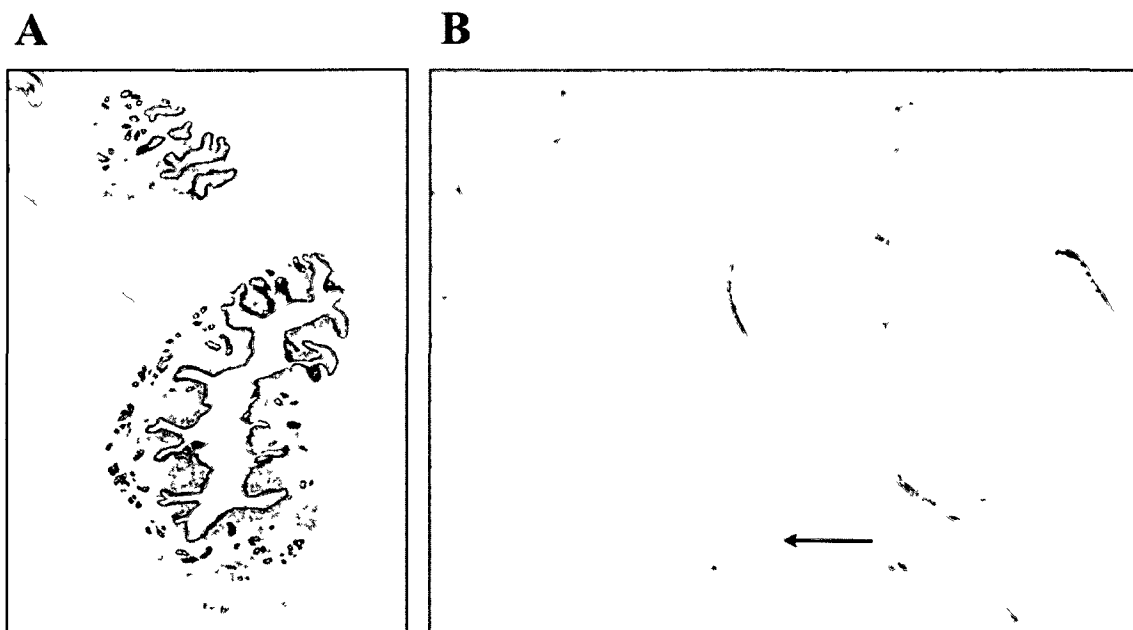


Figure 28: Pax8 is expressed in the mouse uterus and oviduct, but not in the OSE.

(A) Pax8 staining in the epithelium of the normal mouse uterus. (B) Pax8 staining is visible in the normal mouse oviductal epithelium but is absent from the normal mouse ovary, notably the OSE (arrow)

its expression in ovarian tumours *in vivo*. To address this, 3 control and 3 E2-treated tgCAG-LS-TAg primary tumours were examined for *Pax8* mRNA expression by Q-PCR. Though not statistically significant, there was a trend for the E2-treated tumours to express more *Pax8* than the control tumours and this expression pattern was identical after *Tag* normalization (**Figure 29A**). However, there was variability among the E2-treated tumours; two tumour samples expressed relatively high *Pax8*, whereas the third expressed the same low level as the control tumours. Examination of H&E stained slides of one of the high *Pax8*-expressing E2 tumours revealed this tumour was quite small and had some normal uterine tissue in and around the tumour. It is possible that the majority of the RNA extracted from this tissue came from normal uterus, which is known to express *Pax8*. Furthermore, a preliminary IHC assessment of one ovarian tumour from each group of tgCAG-LS-TAg mice (control, E2-treated and P4-treated) showed that these tumours, like their corresponding ascites cell lines, did not express any Pax8 (**Figure 29B**). However, it is unclear from these results whether the tgCAG-LS-TAg tumours truly lack Pax8 expression. Given that such a small sample size of tumours was used for this preliminary analysis, it is possible that it is just these three particular tumours that do not express Pax8, or that there is patchy expression of Pax8 that was not detected in these specific sections. Staining a larger panel of tumours for Pax8 would be required in order to make a more definitive statement about this protein's expression in the tgCAG-LS-TAg mice.

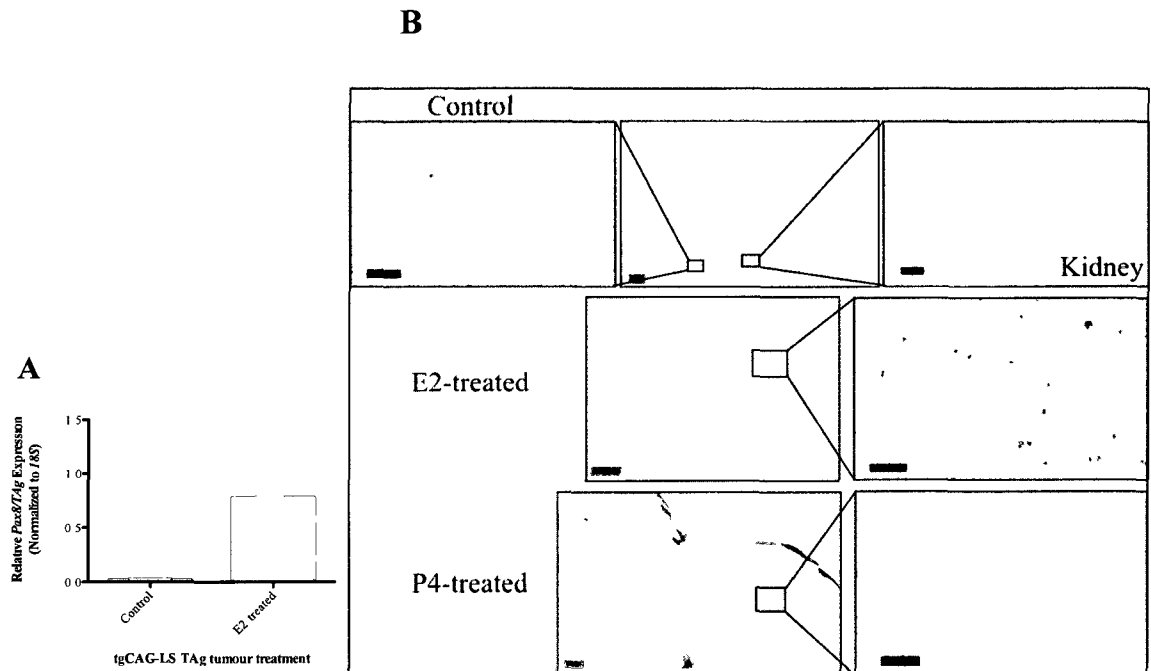


Figure 29: Ovarian tumours from tgCAG-LS-TAg mice do not express *Pax8*. (A)

Relative *Pax8/Tag* mRNA expression in tumours from control and E2-treated tgCAG-LS-TAg mice. Control tumours which are quite large and histologically poorly differentiated do not express much, if any *Pax8*. E2-treated tumours express more *Pax8* than controls, though this is not statistically significant by T test (n = 3 per group). (B) IHC for *Pax8* in control, E2 and P4-treated tgCAG-LS-TAg tumours at low and high magnification. *Pax8* staining is largely absent from all tumours, though some small regions of the control tumour displayed patchy staining (magnification in left hand panel). The control tumour section also includes normal kidney (magnification in right hand panel), an internal positive control; the epithelial cells of the tubules express *Pax8*. The red bars indicate 1 mm and the black bars indicate 100 μ m.

Since human ovarian cancers express PAX8 (Bowen *et al.*, 2007) and sometimes have involvement of other tissues in the reproductive tract such as the fallopian tubes (Salvador *et al.*, 2009), we also wanted to examine Pax8 protein expression in our tgMISIIR-TAg model of EOC which fits this particular pattern of tumour distribution more closely than the tgCAG-LS-TAg model. In tgMISIIR-TAg mice, *TAg* expression is driven by the MISIIR promoter, which is expressed in the OSE, oviduct (equivalent to the human fallopian tubes), and uterus, meaning that tumourigenesis could occur at any or all of these sites. While tgMISIIR-TAg mice develop primary ovarian tumours with an average endpoint of 108 days of age, ovariectomized animals also develop tumours, though these are presumably forming from the uterus or oviduct. Furthermore, ovariectomized mice have an average endpoint of 200 days, suggesting that ovarian tumours are the fastest growing and predominant source of malignant tissue in this transgenic mouse model. A panel of eight ovarian tumours from tgMISIIR-TAg mice was assessed for Pax8 protein expression by IHC. Unlike in the tgCAG-LS-TAg tumours, most of these ovarian tumours expressed some Pax8, though staining was patchy throughout the tumours (**Figure 30A**). Eight tumours from ovariectomized tgMISIIR-TAg mice were also stained with an antibody against Pax8, which revealed that these tumours were more Pax8 positive than the tumours from non-ovariectomized mice. Interestingly, Pax8 staining usually appeared to be localized to regions of papillary differentiation, particularly in the tumours from ovariectomized mice (**Figure 30B**). However, this observation was not formally quantified. It would be interesting to stain the tgMISIIR-TAg tumours for cytokeratins to determine whether there is a correlation

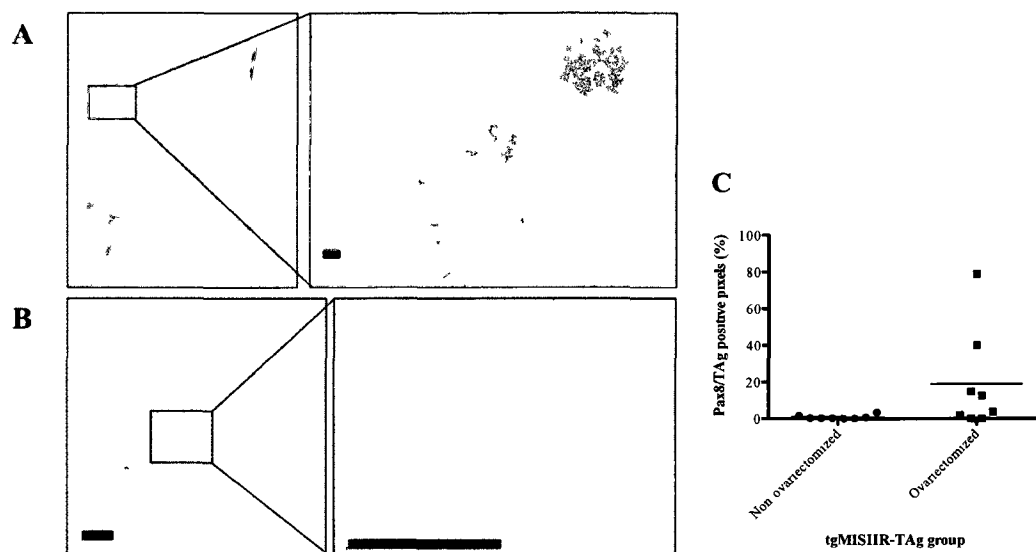


Figure 30: Pax8 expression in tumours from tgMISIIR-TAg mice. (A) A primary tumour from a non-ovariectomized tgMISIIR-TAg mouse sacrificed at 142 days of age exhibits patchy Pax8 staining by IHC. The panel on the right shows the magnification of a portion of the tumour with intense Pax8 staining. The black bar represents 100 μ m. (B) A secondary tumour from a 212 day old tgMISIIR-TAg transgenic mouse whose primary ovarian tumours, bursa and oviduct had been removed at 55 days of age, stained with Pax8. The panel on the right shows the magnification of a tumour section showing regions with papillary structures and intense Pax8 staining. The black bar represents 1 mm. (C) Tumours from ovariectomized tgMISIIR-TAg mice express more Pax8 on average than tumours from non-ovariectomized tgMISIIR-TAg mice, though this is not statistically significant by T test ($p = 0.09$). Positive pixels for Pax8 and TAg were counted and expressed as a percentage of the total number of pixels per ScanScope image. The ratios of Pax8 % positive pixels to TAg % positive pixels per tumour, expressed as percentages, are shown here (Mean 0.9042% for the controls and 18.98% for the ovariectomized mice, $n = 8$ per group).

between Pax8 staining and the epithelial components of these tumours, and whether the proportion of epithelial differentiation differs between tumours from ovariectomized and non-ovariectomized mice. Since Pax8 is expressed in normal uterus and oviduct epithelium, it was important to eliminate the possibility that the Pax8 staining was just coming from the untransformed uterine/oviductal tissue. Serial sections of all tumours were stained with Pax8 and TAg and slides were scanned with the ScanScope. Following visual examination and exclusion of morphologically normal tissue from further analysis, positive pixels (brown staining) were quantified using the ScanScope software, allowing for the normalization of Pax8 to TAg and the identification of the proportion of TAg positive pixels that are also Pax8 positive on serial slides. This showed that there was a higher percentage of TAg positive pixels (tumour cells) that were also Pax8 positive in the tumours from ovariectomized tgMISIIR-TAg mice (mean 18.98%) than in the non-ovariectomized mice (mean 0.9042%) (**Figure 30C**). This suggests that reproductive tract tumours, which have a longer latency than ovarian tumours, also express more Pax8 than ovarian tumours.

CHAPTER 4: DISCUSSION

An important distinction of human ovarian cancer when compared to most other cancers is the tumours' propensity to take on differentiated phenotypes that are much more defined than the putative tissue of origin, the OSE. Normal OSE is a simple squamous to columnar epithelium, whereas the subtypes of human ovarian cancer are histologically quite complex and resemble normal epithelial structures in the female reproductive tract, such as those of the fallopian tubes, uterus, and cervix. The etiology of EOC remains unclear, and the genetic pathways that are involved to initiate and drive the differentiation of the tumours into their distinct histotypes have not been elucidated. Aberrant expression of certain developmental genes that are involved in the normal embryonic differentiation of the female reproductive tract, including the 5' *Hoxa* genes and *Pax8*, have recently been implicated in the differentiation of malignant ovarian tissue; neither HOXA7 nor PAX8 are expressed in normal human OSE, yet both are expressed in well differentiated human ovarian cancers (Cheng *et al.*, 2005; Bowen *et al.*, 2007). Furthermore, *in vitro* (Naora *et al.*, 2001a) and *in vivo* (Cheng *et al.*, 2005) data suggest that *Hoxa7* is causally responsible for epithelial differentiation in OSE and ovarian cancer. Since our mouse models of ovarian cancer generally develop poorly differentiated tumours, we wanted to investigate whether overexpression of *Hoxa7* would induce epithelial differentiation in ovarian cancer cell lines derived from our mouse models or in normal MOSE, and how this overexpression might affect protein expression or cell behaviour. This investigation specifically focused on the potential effects of

overexpression of *Hoxa7* on cell morphology, growth rates, and expression of E-cadherin and vimentin. While the results of this study indicated that overexpression of *Hoxa7* alone was insufficient to significantly alter any of these parameters, *Pax8* emerged as an interesting candidate to pursue, as its expression was correlated with the presence of papillary differentiation in tgMISIIR-TAg tumours.

As the primary and original objective of this project was to determine the influence of *Hoxa7* expression on ovarian cancer morphology, the initial characterization of the cell lines from the mouse models of ovarian cancer used in this project included the assessment of their morphology *in vitro*. We also wanted to examine whether there were any correlations between cell morphology or behaviour and *Hoxa/Pax* gene expression. There appeared to be a connection between growth rate and the morphology of the cancer cells; the MOSE-RM, which had the most classically fibroblastic phenotype grew the fastest of all the cancer cell lines, with a doubling time of 12.02 hours. Conversely, the MISIIR-TAg cell line grew in very epithelial-like clusters of cobblestone shaped cells, and these cells grew relatively slowly, with a doubling time of 25.70 hours. The ascites cell lines, which had fairly plastic phenotypes that varied with density had intermediate growth rates. This seemed promising because it is known that human patients with low grade ovarian tumours that are well differentiated and have an organized, epithelial-like architecture survive longer than those with high grade, poorly differentiated tumours (Gershenson *et al.*, 2006), and this could potentially be attributed to a slower rate of progression in these well differentiated tumours. However, the ovarian tumours produced by our transgenic mouse models are mainly poorly differentiated and the mice succumb

to their disease around the same time after tumour initiation, even though their cell lines have differing *in vitro* growth rates and morphologies. This suggests that factors in the tumour microenvironment are critical for tumour growth and progression, and may account for certain discrepancies between *in vivo* and *in vitro* tumour cell behaviour. This was an important consideration that lead us to examine the gene expression patterns in cell lines in comparison to their primary tumour counterparts.

Since it has been established that the *Hoxa* genes and *Pax8* are not expressed by normal human OSE but are expressed in human ovarian cancers (Cheng *et al.*, 2005; Bowen *et al.*, 2007), the endogenous levels of *Hoxa* and *Pax8* genes in the normal MOSE and mouse ovarian cancer cell lines were measured by Q-PCR to determine if these findings would be consistent in the mouse. Similarly to what has been reported for human OSE (Cheng *et al.*, 2005), the MOSE do not express any *Hoxa9*, *Hoxa10*, or *Hoxa11* mRNA, and they express extremely low levels of *Hoxa7* and *Pax8* mRNA. All mouse cancer cell lines expressed *Hoxa7*, though there was variation in expression levels among all the lines. This agrees with the observation that *HOXA7* is overexpressed in EOC (Ota *et al.*, 2007). Within each cell line, the expression levels of the other *Hoxa* genes were lower than that of *Hoxa7* relative to *18S*, which agrees with the spatially and temporally ordered expression pattern that is characteristic of the *Hox* genes. Still, the expression levels of all the *Hoxa* genes were more similar to each other within a particular cell line than to the level of those same genes in other cell lines. Similarity in *HOX* gene expression within human ovarian cancer cell lines has also been shown, and, like our findings, the expression pattern of *HOX* genes between cell lines is quite variable (Ota *et*

al., 2009). This group suggested that the variability in *HOX* gene expression might be a consequence of the fact that their cell lines were from different patients, and it has also been well established that ovarian cancer is not a homogeneous disease, but rather that each histotype should be regarded as an independent type of cancer. Furthermore, the fact that the mouse ovarian cancer cell lines used in this study did not express the same levels of the same genes is perhaps not too surprising given that the cell lines looked and behaved differently *in vitro*, and disease progression *in vivo* is quite variable among the models. Although the transgenic mouse models from which the murine cancer cell lines used in this project were derived rely on TAg transformation, tgCAG-LS-TAg mice often develop ascites and metastases, whereas tgMISIIR-TAg mice usually die from large primary tumours, and this is most likely reflected in discrete gene expression profiles of several genes.

Another interesting observation that resulted from this gene expression analysis was that there appeared to be a correlation between *Hoxa7* expression and growth rate in the cancer cell lines; the MOSE-RM cells which proliferated the fastest expressed the most endogenous *Hoxa7*, whereas the MISIIR-TAg cells had the slowest growth rate and expressed very little *Hoxa7*. Furthermore, overexpression of *Hoxa7* again appeared to modestly affect proliferation rates in the cells, as the proportion of GFP+ cells in the mixed population of *Hoxa7*-LV-transduced cells increased slightly over several passages. In the cells for which there were repeat assessments, the doubling times also increased slightly. Though this proliferation rate increase did not appear in the sorted cells, this could be because the assays were only conducted over 96 hours, and given the subtle

increase in cell doubling time, mathematically this would not be enough time to notice a difference in the total number of *Hoxa7* cells. These differences are extremely modest, but there are some published data that also correlate the expression of human *HOXA7* with granulosa cell proliferation *in vivo* (Ota *et al.*, 2006) and with OSE subconfluence *in vitro* (Ota *et al.*, 2009). *Hoxa7* cannot be the only factor influencing cell proliferation rate, because although significant overexpression of *Hoxa7* was achieved in the MISIR-TAg cell line, which had the slowest proliferation rate of all the cell lines and a low endogenous level of *Hoxa7* mRNA, its proliferation rate was not increased dramatically, if at all. *Hoxa7* overexpression alone had no effect on proliferation, but it is still possible that it may contribute to OSE and ovarian cancer proliferation in combination with other factors.

The causal role of *Hoxa7* in epithelial ovarian cancer differentiation has not been conclusively established. One report showed that well differentiated human ovarian tumours express more *HOXA7* than poorly differentiated tumours (Cheng *et al.*, 2005). However, in our cell lines there was, if anything, an inverse correlation between *Hoxa7* expression and an epithelial phenotype *in vitro*. Furthermore, overexpression of *Hoxa7* in normal MOSE and in mouse ovarian cancer cell lines did not induce the acquisition of an epithelial morphology. This lead us to conclude that *Hoxa7* alone cannot enhance epithelial differentiation in mouse ovarian cancer cells *in vitro*. Another group has shown that *HOXA7* expression in human ovarian tumours was variable and unrelated to tumour histotype or grade (Ota *et al.*, 2007), likewise suggesting that *HOXA7* expression is not the sole determinant of ovarian tumour differentiation, if it is involved in this process at

all. In addition, the correlation between HOXA7 and well differentiated tumours reported by Cheng *et al.* seems to conflict with the published links between *HOXA7* and increased proliferation because of the more indolent nature of low grade compared to high grade ovarian tumours. Although all the tumours from our mouse models are poorly differentiated for the most part, and protein expression analysis of *Hoxa7 in situ* was hindered by difficulties with the commercially available *Hoxa7* antibodies, it could be valuable to determine whether *Hoxa7* protein is expressed in these tumours, at any level or in association with differentiated cells.

In addition to cell morphology, changes in expression of epithelial and mesenchymal markers are another indication of differentiation, so we examined the expression of E-cadherin and vimentin in all cell lines with and without *Hoxa7* overexpression. In most carcinomas, E-cadherin is lost from the epithelial tissue of origin as cancers develop; however, this loss of E-cadherin is not seen in early ovarian cancer. On the contrary, it has previously been reported that human OSE express little to no E-cadherin, and expression of this protein is gained in ovarian cancer (Maines-Bandiera and Auersperg 1997; Sundfeldt *et al.*, 1997). Likewise, we found that normal MOSE expressed very low levels of E-cadherin and all the ovarian cancer cell lines expressed higher levels of E-cadherin. Vimentin was also expressed in the MOSE, but among the cancer cell lines its expression was somewhat variable.

In humans, HOXA7 and E-cadherin are newly expressed in ovarian inclusion cysts, although it remains unclear whether one causes expression of the other. There was no significant influence of *Hoxa7* overexpression on E-cadherin or vimentin levels in any

of our murine cell lines. These data are in contrast with two publications detailing the effects of ectopic *Hoxa7* expression on inducing a differentiated morphology in human or mouse OSE. In these papers, transfection of these cells with *Hoxa7* was associated with the acquisition of E-cadherin in culture (Naora *et al.*, 2001a) and maintenance of its expression in tumours (Cheng *et al.*, 2005). All of the cancer cell lines used for this study expressed E-cadherin regardless of their *Hoxa7* status. However, unpublished IHC data from other members of our lab showed that E-cadherin staining in the tgCAG-LS-TAg tumours was the strongest in the areas of the tumours that contained papillary structures, and pan-cytokeratin staining (indicative of epithelium) was extremely variable within and between tgMISIIR-TAg tumours, suggesting that there may also be regions within these tumours that could also be positive for E-cadherin. Though there appeared to be no correlation between *Hoxa7* mRNA expression and E-cadherin protein levels in the mouse ovarian cancer cell lines, it would be extremely valuable to determine whether there is co-localization of *Hoxa7* and E-cadherin within the tgCAG-LS-TAg and tgMISIIR-TAg tumours. This would clarify whether there is a relationship between epithelial differentiation and *Hoxa7* in our mouse models of EOC. However, the fact that all cell lines expressed E-cadherin irrespective of *Hoxa7* levels suggests that though the expression of these proteins in human inclusion cysts may be related, *Hoxa7* does not directly regulate the expression of E-cadherin in several mouse ovarian cancer cell lines *in vitro*. This could be a species or strain-specific interaction which is simply inapplicable to mice on the FVB/N background, whereas the mice in Cheng *et al.* were C57BL6, or it could be explained by differing microenvironments. Inclusion cysts are trapped within

the ovarian stroma and therefore are potentially exposed to different growth factors and OSE-stromal cell interactions than cells in a cultured monolayer would experience. The importance of the ovarian microenvironment on cell behaviour has been supported by evidence from our lab that tgCAG-LS-TAg MOSE do not respond to AdCre-mediated recombination the same way *in vivo* and *in vitro*. tgCAG-LS-TAg MOSE taken from a mouse that has never been exposed to AdCre cannot be transformed by addition of AdCre to these cells in culture, but 82% of the tgCAG-LS-TAg mice that surgically receive AdCre under the ovarian bursa develop tumours.

One point to keep in mind when considering the outcome of the *Hoxa7* overexpression experiments is that though a significant increase in *Hoxa7* mRNA was confirmed for all transduced cell lines, there was variable GFP expression in these cells. GFP is downstream of *Hoxa7* in the lentiviral construct, so either the IRES is not 100% efficient in linking GFP expression to *Hoxa7* expression, or *Hoxa7* is not always expressed in all of the cells at the same time. Furthermore, because we did not have a *Hoxa7* antibody that worked well, *Hoxa7* protein expression was not directly confirmed. It is possible that *Hoxa7* was transcribed but not efficiently translated in our cells for some reason, which could also account for the lack of an observed phenotype in the transduced cells.

While *Hoxa7* mRNA overexpression did not affect either cell morphology or E-cadherin levels in any of the cell lines, these features were altered in some of the cell lines by EGF. We initially directed our attention to the effects of EGF in the culture media on *Hoxa7* expression in all cell lines since EGF induced *Hoxa7* in cultured keratinocytes

(La Celle and Polakowska, 2001), but *Hoxa7* levels were the same in EGF- and EGF+ cells. However, the MOSE and some mouse ovarian cancer cells maintained without EGF looked rounder, flatter, and more spread out than those cultured with EGF in the media. It has been reported that human OSE maintained without EGF looked cobblestone-like, whereas the addition of EGF and hydrocortisone made the cells acquire a more fibroblastic phenotype (Salamanca *et al.*, 2004; Ahmed *et al.*, 2006). The cells in this study were not treated with hydrocortisone so it is conceivable that their morphology would not exactly mirror those published previously, but there was definitely a morphological difference in both the normal MOSE and ovarian cancer EGF+ and EGF- cell lines. Furthermore, this morphological change was associated with a difference in E-cadherin expression; cells cultured with EGF expressed less E-cadherin than those cultured without EGF. These *in vitro* data agree with what is known about EGF-mediated epithelial to mesenchymal transition (EMT) in both normal OSE and late stage ovarian cancer. EGF induces EMT in human OSE in culture and activates several downstream pathways that promote cell motility and invasion, including the extracellular signal-regulated kinase (Erk) and integrin-linked kinase (ILK) pathways (Ahmed *et al.*, 2006). One result of EMT in advanced ovarian cancer is the downregulation of E-cadherin, and the expression of E-cadherin in ascites cells that have disseminated into the peritoneum is greatly decreased relative to primary solid ovarian tumours (Veatch *et al.*, 1994). This event has been shown to be directly regulated by EGF, via upregulation of Matrix Metalloproteinase-9 (Cowden Dahl *et al.*, 2008).

Though EGF affected some characteristics of the cells, it did not affect *Hoxa7* expression. Therefore, other factors must account for the difference in *Hoxa7* expression that was observed between the cultured ascites cell lines and the primary tumours from the same mouse models of ovarian cancer. The two obvious options were that this was an effect of culture, specifically that adherent culture of originally free-floating ascites cells was affecting gene expression, or that the observed difference in *Hoxa7* between ascites and tumours was reflective of differences between these cells *in vivo*. To address the first possibility, the three ascites cell lines were cultured in suspension conditions. All three lines easily formed tightly packed sphere-like cell clusters and survived in suspension, however this had no effect on *Hoxa7* expression. This is perhaps because while the cells were not attached to the plate, they were still firmly attached to each other; the clusters had to be incubated in trypsin for a few minutes to disaggregate them because simple pipetting was insufficient to do this. Therefore, it might be more appropriate to look at the expression of *Hoxa7* in single cell suspensions that can neither attach to a substrate nor to other cells in order to elucidate whether there is any correlation between *Hoxa7* and attachment.

To address the possibility that ascites and tumours truly differentially expressed *Hoxa7*, mRNA levels of this gene in paired ascites and tumour samples from SCID mouse xenograft studies were compared. This revealed that there was a difference between ascites and tumours, but contrary to what was seen previously in the comparison between the cell lines and the primary tumours, SCID ascites expressed less *Hoxa7* than the SCID tumours. It also showed that, upon treatment with estrogen, the mean *Hoxa7*

levels decreased by more than half in both the ascites and tumours from SCIDs xenografted with MASC1 cells. This indicated a potential suppressive effect of estrogen on *Hoxa7*. While it is unknown whether *Hoxa7* is regulated by estrogen in the ovary, expression of *Hoxa10* in the mouse uterus is suppressed by estrogen (Ma *et al.*, 1998). Data from our lab indicate that estrogen treatment increases both preneoplastic lesions in normal mouse ovaries and areas of papillary differentiation in mouse ovarian tumours, and if indeed estrogen suppresses *Hoxa7*, this would be more evidence to suggest that *Hoxa7* alone does not induce epithelial differentiation in mouse ovarian tumours. To determine if there is an interaction between estrogen and *Hoxa7* in the cell lines used in this study, it would be useful to treat them with estrogen and examine them for any changes in *Hoxa7* expression. The findings that SCID ascites express less *Hoxa7* than their matched tumours also provided support for the notion that the increased *Hoxa7* in the ascites cell lines was the result of an as yet unidentified culture condition.

We investigated the expression of *Pax8* in our mouse ovarian cancer cell lines and tumours because, as with *Hoxa7*, this gene is absent from normal human OSE but is expressed in normal fallopian tube epithelium and ovarian tumours (Bowen *et al.*, 2007). Since *Pax8* normally contributes to the development of the Müllerian duct epithelium (Kobayashi and Behringer, 2003), it presents itself as another interesting potential player in driving the epithelial differentiation of EOC. Like human OSE, the MOSE did not express *Pax8* mRNA or protein. However, its expression in tumours was variable among the models and seemed to be correlated with the degree of differentiation or the number of papillary structures. Unlike in human ovarian tumours, neither the ascites cell lines nor

a small sample of primary tumours from the tgCAG-LS-TAg mouse model of ovarian cancer expressed *Pax8* mRNA or protein. One explanation for this may be that the human ovarian tumours that were PAX8 positive were well differentiated enough to classify them by histotype (Bowen *et al.*, 2007), whereas the tgCAG-LS-TAg tumours were poorly differentiated. It is also possible that such a small sample size of three tumours was not representative of Pax8 expression in all the tumours, or that there was patchy expression within the tumours that was not captured in the particular sections that were examined here. The tgMISIIR-TAg primary ovarian tumours are also poorly differentiated, for the most part, yet they displayed patchy positive staining for Pax8. These areas of positive staining were small relative to the entire size of the tumour, and when they did occur, they were usually areas that contained papillary structures. Therefore, it is possible that transformed MOSE that acquire Pax8 expression become more differentiated in phenotype. However, unlike in the tgCAG-LS-TAg model, TAg expression is driven by the MISIIR promoter, which is active in the oviduct and uterus as well as the OSE, so there may have been some contribution to the ovarian tumour from these other tissues, which could also be the Pax8-positive areas. This is supported by the observation that the secondary tumours from tgMISIIR-TAg ovariectomized mice were more Pax8-positive than the primary ovarian tumours from the non-ovariectomized animals. In this model, TAg expression is seen in the oviduct epithelium and stroma and in the epithelium of the uterus, but Pax8 is restricted to the epithelium of both of these tissues. Although the tumours from both groups of tgMISIIR-TAg mice expressed the same amount of TAg, the secondary tumours in the ovariectomized mice may have arisen

from both stromal and epithelial cells of the oviduct and possibly the contribution of these two cell types (stromal or epithelial) to the emerging tumours may have been reflected in the degree of Pax8 relative to TAG staining.

The observed association between epithelial differentiation and *Pax8* expression in the tgMISIIR-TAg tumours was not seen in the MOSE-RM cells, the only cell line that expressed a significantly higher amount of *Pax8* mRNA than normal MOSE. This cell line was derived from immortalized but non-tumourigenic MOSE that had been transformed by infection with retroviral constructs expressing *K-ras*^{G12D} and *c-myc*, so there may have been some influence of the activation of these genes on *Pax8* expression that is not present in the transgenic mouse models. The relationship between *Pax8* and *K-ras*^{G12D} or *c-myc* in the MOSE-RM has not been studied, and a direct interaction between them would not necessarily be expected, given that these oncogenes are thought to contribute to the extremely aggressive and generally poorly differentiated nature of the tumours produced by these cells. However, it is known that the expression of *c-Myc* and *PAX8* in human thyroid carcinomas is not mutually exclusive (Basolo *et al.*, 2002), therefore it is possible that there is some interaction between these two genes in certain cancer cells. Furthermore, very little histological analysis and no IHC has been performed on the tumours produced by MOSE-RM cells in nude or SCID mice, so it remains unknown whether there are epithelial components of the tumours and if they express Pax8 in those regions. There have been other suggested roles for aberrant *PAX8* expression in cancer, such as promoting cell survival by transcriptionally repressing *p53*

and activating *bcl-2* (Stuart *et al.*, 1995; Hewitt *et al.*, 1997), which could also account for the increased expression of *Pax8* in the highly aggressive MOSE-RM cells.

As with the observations for *Hoxa7*, there are differences in *Pax8* expression between human ovarian tumours and those from our mouse models, and there could be a few reasons for this. It is possible and has been suggested several times that what are considered to be “ovarian” tumours are actually primary fallopian tube tumours that have invaded the ovary (Crum *et al.*, 2007; Salvador *et al.*, 2009). This was suggested for several reasons, including the fact that serous tumours of the fallopian tube histologically resemble serous ovarian tumours, and they also express many of the markers that serous ovarian tumours do, including PAX8 (Roh *et al.*, 2009). However, PAX8 is also expressed in the OSE of inclusion cysts which display more evidence of epithelial differentiation than surface OSE (Bowen *et al.*, 2007), suggesting that the OSE cannot yet be ruled out as the source of ovarian cancer. It is also possible that human and mouse ovarian tumours simply do not express the same genes; human ovarian tumours express PAX8 and have a well differentiated histological phenotype, whereas the current mouse models develop ovarian tumours that may not be predisposed to express this protein and are not well differentiated. tgMISIIR-TAg mouse tumours that do show evidence of papillary differentiation widely express Pax8, though notably these regions are more prevalent in non-ovarian tumours. It is unclear whether PAX8 has a causal influence on EOC differentiation; transformed OSE may acquire PAX8 expression and become more differentiated as a result, or PAX8 expression may be a consequence of the ovarian tumours containing regions of epithelial differentiation, which may or may not be from

tumourigenic fallopian tube cells that have maintained some of their original architecture. It would be interesting to see if this gene was indeed responsible for epithelial ovarian tumour differentiation by examining whether it could induce differentiation in our mouse models by the same methods of overexpression that were performed in this study with *Hoxa7*. Looking at *Pax8* would perhaps be more promising than *Hoxa7* since neither the MOSE nor the cell lines endogenously express *Pax8*. If *Pax8* overexpression was not sufficient to enhance the differentiation of ovarian cancer cells either *in vitro* or *in vivo* (especially those from the tgCAG-LS-TAg tumours that are known to come from the OSE), then that would also add more weight to the argument that ovarian cancer likely originates elsewhere in the reproductive tract.

The original hypothesis that *Hoxa7* is centrally involved in promoting epithelial differentiation was not supported by the results in this project. There were certain results that were consistent between what has been reported for humans and what was observed for the mouse, such as the similarities in *Hoxa* and *Pax8* gene expression in the OSE of both species. However, unlike in human EOC, there did not seem to be a correlation between expression of any of the *Hoxa* genes and differentiation. In our mouse models of ovarian cancer, *Hoxa7* alone did not cause epithelial differentiation, but its interaction with the other *Abd-B*-like *Hoxa* genes has not yet been investigated, and there remains the possibility that multiple *Hoxa* genes are involved in this process. Furthermore, data from this project suggest that *Hoxa7* might play a small role in promoting cell proliferation or regulating attachment of cancer cells. *Pax8* may be a more interesting gene candidate in the study of EOC differentiation, as its expression was correlated with

papillary differentiation in tumours from ovariectomized tgMISIIR-TAg mice. The potential of this gene to cause this type of differentiation in our transgenic mouse models of ovarian cancer warrants further investigation.

REFERENCES

- Abate-Shen C. 2002. Deregulated homeobox gene expression in cancer: Cause or consequence? *Nat Rev Cancer* 2(10):777-85.
- Abdel-Fattah R, Xiao A, Bomgardner D, Pease CS, Lopes MB, Hussaini IM. 2006. Differential expression of HOX genes in neoplastic and non-neoplastic human astrocytes. *J Pathol* 209(1):15-24.
- Abe M, Hamada J, Takahashi O, Takahashi Y, Tada M, Miyamoto M, Morikawa T, Kondo S, Moriuchi T. 2006. Disordered expression of HOX genes in human non-small cell lung cancer. *Oncol Rep* 15(4):797-802.
- Aberdam D, Negreanu V, Sachs L, Blatt C. 1991. The oncogenic potential of an activated hox-2.4 homeobox gene in mouse fibroblasts. *Mol Cell Biol* 11(1):554-7.
- Ahmed N, Maines-Bandiera S, Quinn MA, Unger WG, Dedhar S, Auersperg N. 2006. Molecular pathways regulating EGF-induced epithelio-mesenchymal transition in human ovarian surface epithelium. *Am J Physiol Cell Physiol* 290(6):C1532-42.
- Auersperg N, Siemens CH, Myrdal SE. 1984. Human ovarian surface epithelium in primary culture. *In Vitro* 20(10):743-55.
- Auersperg N, Maines-Bandiera SL, Dyck HG, Kruk PA. 1994. Characterization of cultured human ovarian surface epithelial cells: Phenotypic plasticity and premalignant changes. *Lab Invest* 71(4):510-8.
- Auersperg N, Wong AS, Choi KC, Kang SK, Leung PC. 2001. Ovarian surface epithelium: Biology, endocrinology, and pathology. *Endocr Rev* 22(2):255-88.
- Bahrani-Mostafavi Z, Tickle TL, Zhang J, Bennett KE, Vachris JC, Spencer MD, Mostafavi MT, Tait DL. 2008. Correlation analysis of HOX, ErbB and IGFBP family gene expression in ovarian cancer. *Cancer Invest* 26(10):990-8.
- Basolo F, Giannini R, Toniolo A, Casalone R, Nikiforova M, Pacini F, Elisei R, Miccoli P, Berti P, Faviana P, et al. 2002. Establishment of a non-tumorigenic papillary thyroid cell line (FB-2) carrying the RET/PTC1 rearrangement. *Int J Cancer* 97(5):608-14.
- Bell DA and Scully RE. 1994. Early de novo ovarian carcinoma. A study of fourteen cases. *Cancer* 73(7):1859-64.
- Benson GV, Lim H, Paria BC, Satokata I, Dey SK, Maas RL. 1996. Mechanisms of reduced fertility in hoxa-10 mutant mice: Uterine homeosis and loss of maternal hoxa-10 expression. *Development* 122(9):2687-96.
- Blaustein A and Lee H. 1979. Surface cells of the ovary and pelvic peritoneum: A histochemical and ultrastructure comparison. *Gynecol Oncol* 8(1):34-43.

- Block K, Kardana A, Igarashi P, Taylor HS. 2000. In utero diethylstilbestrol (DES) exposure alters hox gene expression in the developing mullerian system. *FASEB J* 14(9):1101-8.
- Bowen NJ, Logani S, Dickerson EB, Kapa LB, Akhtar M, Benigno BB, McDonald JF. 2007. Emerging roles for PAX8 in ovarian cancer and endosalpingeal development. *Gynecol Oncol* 104(2):331-7.
- Brandenberger AW, Tee MK, Jaffe RB. 1998. Estrogen receptor alpha (ER-alpha) and beta (ER-beta) mRNAs in normal ovary, ovarian serous cystadenocarcinoma and ovarian cancer cell lines: Down-regulation of ER-beta in neoplastic tissues. *J Clin Endocrinol Metab* 83(3):1025-8.
- Canadian Cancer Society's Steering Committee: Canadian Cancer Statistics (2009). Toronto: Canadian Cancer Society, 2009.
- Cannistra SA. 2004. Cancer of the ovary. *N Engl J Med* 351(24):2519-29.
- Care A, Silvani A, Meccia E, Mattia G, Peschle C, Colombo MP. 1998. Transduction of the SkBr3 breast carcinoma cell line with the HOXB7 gene induces bFGF expression, increases cell proliferation and reduces growth factor dependence. *Oncogene* 16(25):3285-9.
- Chen F and Capecchi MR. 1999. Paralogous mouse hox genes, Hoxa9, Hoxb9, and Hoxd9, function together to control development of the mammary gland in response to pregnancy. *Proc Natl Acad Sci U S A* 96(2):541-6.
- Cheng W, Liu J, Yoshida H, Rosen D, Naora H. 2005. Lineage infidelity of epithelial ovarian cancers is controlled by HOX genes that specify regional identity in the reproductive tract. *Nat Med* 11(5):531-7.
- Cho KR and Shih I. 2009. Ovarian cancer. *Annu Rev Pathol* 4:287-313.
- Cho KW, Morita EA, Wright CV, De Robertis EM. 1991. Overexpression of a homeodomain protein confers axis-forming activity to uncommitted xenopus embryonic cells. *Cell* 65(1):55-64.
- Connolly DC, Bao R, Nikitin AY, Stephens KC, Poole TW, Hua X, Harris SS, Vanderhyden BC, Hamilton TC. 2003. Female mice chimeric for expression of the simian virus 40 TAg under control of the MISIR promoter develop epithelial ovarian cancer. *Cancer Res* 63(6):1389-97.
- Cowden Dahl KD, Symowicz J, Ning Y, Gutierrez E, Fishman DA, Adley BP, Stack MS, Hudson LG. 2008. Matrix metalloproteinase 9 is a mediator of epidermal growth factor-dependent e-cadherin loss in ovarian carcinoma cells. *Cancer Res* 68(12):4606-13.
- Crum CP, Drapkin R, Miron A, Ince TA, Muto M, Kindelberger DW, Lee Y. 2007. The distal fallopian tube: A new model for pelvic serous carcinogenesis. *Curr Opin Obstet Gynecol* 19(1):3-9.

- Cuatrecasas M, Villanueva A, Matias-Guiu X, Prat J. 1997. K-ras mutations in mucinous ovarian tumors: A clinicopathologic and molecular study of 95 cases. *Cancer* 79(8): 1581-6.
- Davies BR, Finnigan DS, Smith SK, Ponder BA. 1999. Administration of gonadotropins stimulates proliferation of normal mouse ovarian surface epithelium. *Gynecol Endocrinol* 13(2):75-81.
- Davies BR, Steele IA, Edmondson RJ, Zwolinski SA, Saretzki G, von Zglinicki T, O'Hare MJ. 2003. Immortalisation of human ovarian surface epithelium with telomerase and temperature-sensitive SV40 large T antigen. *Exp Cell Res* 288(2): 390-402.
- De Souza Setubal Destro MF, Bitu CC, Zecchin KG, Graner E, Lopes MA, Kowalski LP, Coletta RD. 2010. Overexpression of HOXB7 homeobox gene in oral cancer induces cellular proliferation and is associated with poor prognosis. *Int J Oncol* 36(1):141-9.
- Dolle P and Duboule D. 1989. Two gene members of the murine HOX-5 complex show regional and cell-type specific expression in developing limbs and gonads. *EMBO J* 8(5):1507-15.
- Doraiswamy V, Parrott JA, Skinner MK. 2000. Expression and action of transforming growth factor alpha in normal ovarian surface epithelium and ovarian cancer. *Biol Reprod* 63(3):789-96.
- Drapkin R, Crum CP, Hecht JL. 2004. Expression of candidate tumor markers in ovarian carcinoma and benign ovary: Evidence for a link between epithelial phenotype and neoplasia. *Hum Pathol* 35(8):1014-21.
- Duboule D. 1991. Patterning in the vertebrate limb. *Curr Opin Genet Dev* 1(2):211-6.
- Eva A, Robbins KC, Andersen PR, Srinivasan A, Tronick SR, Reddy EP, Ellmore NW, Galen AT, Lautenberger JA, Papas TS, et al. 1982. Cellular genes analogous to retroviral onc genes are transcribed in human tumour cells. *Nature* 295(5845):116-9.
- Fleming JS, McQuillan HJ, Millier MJ, Beaugie CR, Livingstone V. 2007. E-cadherin expression and bromodeoxyuridine incorporation during development of ovarian inclusion cysts in age-matched breeder and incessantly ovulated CD-1 mice. *Reprod Biol Endocrinol* 5:14.
- Friedmann Y, Daniel CA, Strickland P, Daniel CW. 1994. Hox genes in normal and neoplastic mouse mammary gland. *Cancer Res* 54(22):5981-5.
- Fujimoto J, Ichigo S, Hirose R, Sakaguchi H, Tamaya T. 1997. Expression of E-cadherin and alpha- and beta-catenin mRNAs in ovarian cancers. *Cancer Lett* 115(2):207-12.
- Gaetje R, Holtrich U, Engels K, Kissler S, Rody A, Karn T, Kaufmann M. 2007. Endometriosis may be generated by mimicking the ontogenetic development of the female genital tract. *Fertil Steril* 87(3):651-6.

- Garcia-Fernandez J. 2005. The genesis and evolution of homeobox gene clusters. *Nat Rev Genet* 6(12):881-92.
- Garin E, Lemieux M, Coulombe Y, Robinson GW, Jeannotte L. 2006. Stromal Hoxa5 function controls the growth and differentiation of mammary alveolar epithelium. *Dev Dyn* 235(7):1858-71.
- Gatcliffe TA, Monk BJ, Planutis K, Holcombe RF. 2008. Wnt signaling in ovarian tumorigenesis. *Int J Gynecol Cancer* 18(5):954-62.
- Gershenson DM, Sun CC, Lu KH, Coleman RL, Sood AK, Malpica A, Deavers MT, Silva EG, Bodurka DC. 2006. Clinical behavior of stage II-IV low-grade serous carcinoma of the ovary. *Obstet Gynecol* 108(2):361-8.
- Geyer JT, Lopez-Garcia MA, Sanchez-Estevez C, Sarrio D, Moreno-Bueno G, Franceschetti I, Palacios J, Oliva E. 2009. Pathogenetic pathways in ovarian endometrioid adenocarcinoma: A molecular study of 29 cases. *Am J Surg Pathol* 33(8):1157-63.
- Gilks CB. 2010. Molecular abnormalities in ovarian cancer subtypes other than high-grade serous carcinoma. *J Oncol* 2010:740968.
- Gruss P and Kessel M. 1991. Axial specification in higher vertebrates. *Curr Opin Genet Dev* 1(2):204-10.
- Guioli S, Sekido R, Lovell-Badge R. 2007. The origin of the mullerian duct in chick and mouse. *Dev Biol* 302(2):389-98.
- Hanawa H, Kelly PF, Nathwani AC, Persons DA, Vandergriff JA, Hargrove P, Vanin EF, Nienhuis AW. 2002. Comparison of various envelope proteins for their ability to pseudotype lentiviral vectors and transduce primitive hematopoietic cells from human blood. *Mol Ther* 5(3):242-51.
- Hashimoto R. 2003. Development of the human mullerian duct in the sexually undifferentiated stage. *Anat Rec A Discov Mol Cell Evol Biol* 272(2):514-9.
- Hess V, A'Hern R, Nasiri N, King DM, Blake PR, Barton DP, Shepherd JH, Ind T, Bridges J, Harrington K, et al. 2004. Mucinous epithelial ovarian cancer: A separate entity requiring specific treatment. *J Clin Oncol* 22(6):1040-4.
- Hewitt SM, Hamada S, Monarres A, Kottical LV, Saunders GF, McDonnell TJ. 1997. Transcriptional activation of the bcl-2 apoptosis suppressor gene by the paired box transcription factor PAX8. *Anticancer Res* 17(5A):3211-5.
- Hogberg T, Glimelius B, Nygren P, SBU-group. Swedish Council of Technology Assessment in Health Care. 2001. A systematic overview of chemotherapy effects in ovarian cancer. *Acta Oncol* 40(2-3):340-60.
- Hsieh-Li HM, Witte DP, Weinstein M, Branford W, Li H, Small K, Potter SS. 1995. Hoxa 11 structure, extensive antisense transcription, and function in male and female fertility. *Development* 121(5):1373-85.

- Ismail RS, Cada M, Vanderhyden BC. 1999. Transforming growth factor-beta regulates kit ligand expression in rat ovarian surface epithelial cells. *Oncogene* 18(33): 4734-41.
- Jiang F, Saunders BO, Haller E, Livingston S, Nicosia SV, Bai W. 2003. Conditionally immortal ovarian cell lines for investigating the influence of ovarian stroma on the estrogen sensitivity and tumorigenicity of ovarian surface epithelial cells. *In Vitro Cell Dev Biol Anim* 39(7):304-12.
- Kmita M and Duboule D. 2003. Organizing axes in time and space; 25 years of colinear tinkering. *Science* 301(5631):331-3.
- Kobayashi A and Behringer RR. 2003. Developmental genetics of the female reproductive tract in mammals. *Nat Rev Genet* 4(12):969-80.
- Kobel M, Kalloger SE, Carrick J, Huntsman D, Asad H, Oliva E, Ewanowich CA, Soslow RA, Gilks CB. 2009. A limited panel of immunomarkers can reliably distinguish between clear cell and high-grade serous carcinoma of the ovary. *Am J Surg Pathol* 33(1):14-21.
- Korner M, Burckhardt E, Mazzucchelli L. 2005. Different proportions of aneusomic cells in ovarian inclusion cysts associated with serous borderline tumours and serous high-grade carcinomas support different pathogenetic pathways. *J Pathol* 207(1): 20-6.
- Kroon E, Kros J, Thorsteinsdottir U, Baban S, Buchberg AM, Sauvageau G. 1998. Hoxa9 transforms primary bone marrow cells through specific collaboration with Meis1a but not Pbx1b. *EMBO J* 17(13):3714-25.
- Kros J, Baban S, Kros G, Rozenfeld S, Largman C, Sauvageau G. 1998. Cellular proliferation and transformation induced by HOXB4 and HOXB3 proteins involves cooperation with PBX1. *Oncogene* 16(26):3403-12.
- Kruk PA, Maines-Bandiera SL, Auersperg N. 1990. A simplified method to culture human ovarian surface epithelium. *Lab Invest* 63(1):132-6.
- La Celle PT and Polakowska RR. 2001. Human homeobox HOXA7 regulates keratinocyte transglutaminase type 1 and inhibits differentiation. *J Biol Chem* 276(35):32844-53.
- Lappin TR, Grier DG, Thompson A, Halliday HL. 2006. HOX genes: Seductive science, mysterious mechanisms. *Ulster Med J* 75(1):23-31.
- Lau KM, Mok SC, Ho SM. 1999. Expression of human estrogen receptor-alpha and -beta, progesterone receptor, and androgen receptor mRNA in normal and malignant ovarian epithelial cells. *Proc Natl Acad Sci U S A* 96(10):5722-7.
- Laviolette LA, Garson K, Macdonald EA, Senterman MK, Courville K, Crane CA, Vanderhyden BC. 2010. 17 β -estradiol accelerates tumor onset and decreases survival in a transgenic mouse model of ovarian cancer. *Endocrinology* 151(3): 929-38.

- Le Mouellic H, Lallemand Y, Brulet P. 1992. Homeosis in the mouse induced by a null mutation in the *hox-3.1* gene. *Cell* 69(2):251-64.
- Lewis EB. 1956. New mutants: Report of E.B. lewis. *Drosoph Inf Serv* 30:76-7.
- Lewis EB. 1978. A gene complex controlling segmentation in *drosophila*. *Nature* 276(5688):565-70.
- Li B, Jin H, Yu Y, Gu C, Zhou X, Zhao N, Feng Y. 2009. HOXA10 is overexpressed in human ovarian clear cell adenocarcinoma and correlates with poor survival. *Int J Gynecol Cancer* 19(8):1347-52.
- Lu JJ, Zheng Y, Kang X, Yuan JM, Lauchlan SC, Pike MC, Zheng W. 2000. Decreased luteinizing hormone receptor mRNA expression in human ovarian epithelial cancer. *Gynecol Oncol* 79(2):158-68.
- Ma L, Benson GV, Lim H, Dey SK, Maas RL. 1998. Abdominal B (AbdB) *hoxa* genes: Regulation in adult uterus by estrogen and progesterone and repression in mullerian duct by the synthetic estrogen diethylstilbestrol (DES). *Dev Biol* 197(2):141-54.
- Maines-Bandiera SL and Auersperg N. 1997. Increased E-cadherin expression in ovarian surface epithelium: An early step in metaplasia and dysplasia? *Int J Gynecol Pathol* 16(3):250-5.
- Mansouri A, Hallonet M, Gruss P. 1996. Pax genes and their roles in cell differentiation and development. *Curr Opin Cell Biol* 8(6):851-7.
- Marquez RT, Baggerly KA, Patterson AP, Liu J, Broaddus R, Frumovitz M, Atkinson EN, Smith DI, Hartmann L, Fishman D, et al. 2005. Patterns of gene expression in different histotypes of epithelial ovarian cancer correlate with those in normal fallopian tube, endometrium, and colon. *Clin Cancer Res* 11(17):6116-26.
- Maulbecker CC and Gruss P. 1993a. The oncogenic potential of deregulated homeobox genes. *Cell Growth Differ* 4(5):431-41.
- Maulbecker CC and Gruss P. 1993b. The oncogenic potential of pax genes. *EMBO J* 12(6):2361-7.
- McAlpine JN, Wiegand KC, Vang R, Ronnett BM, Adamiak A, Kobel M, Kalloger SE, Swenerton KD, Huntsman DG, Gilks CB, et al. 2009. HER2 overexpression and amplification is present in a subset of ovarian mucinous carcinomas and can be targeted with trastuzumab therapy. *BMC Cancer* 9:433.
- McGinnis W, Hart CP, Gehring WJ, Ruddle FH. 1984. Molecular cloning and chromosome mapping of a mouse DNA sequence homologous to homeotic genes of *drosophila*. *Cell* 38(3):675-80.
- Miao J, Wang Z, Provencher H, Muir B, Dahiya S, Carney E, Leong CO, Sgroi DC, Orsulic S. 2007. HOXB13 promotes ovarian cancer progression. *Proc Natl Acad Sci U S A* 104(43):17093-8.

- Milner BJ, Allan LA, Eccles DM, Kitchener HC, Leonard RC, Kelly KF, Parkin DE, Haites NE. 1993. P53 mutation is a common genetic event in ovarian carcinoma. *Cancer Res* 53(9):2128-32.
- Mittal KR, Zeleniuch-Jacquotte A, Cooper JL, Demopoulos RI. 1993. Contralateral ovary in unilateral ovarian carcinoma: A search for preneoplastic lesions. *Int J Gynecol Pathol* 12(1):59-63.
- Moens CB and Selleri L. 2006. Hox cofactors in vertebrate development. *Dev Biol* 291(2):193-206.
- Murdoch WJ. 1994. Ovarian surface epithelium during ovulatory and anovulatory ovine estrous cycles. *Anat Rec* 240(3):322-6.
- Naora H, Montz FJ, Chai CY, Roden RB. 2001a. Aberrant expression of homeobox gene HOXA7 is associated with mullerian-like differentiation of epithelial ovarian tumors and the generation of a specific autologous antibody response. *Proc Natl Acad Sci U S A* 98(26):15209-14.
- Naora H, Yang YQ, Montz FJ, Seidman JD, Kurman RJ, Roden RB. 2001b. A serologically identified tumor antigen encoded by a homeobox gene promotes growth of ovarian epithelial cells. *Proc Natl Acad Sci U S A* 98(7):4060-5.
- Nossov V, Amneus M, Su F, Lang J, Janco JM, Reddy ST, Farias-Eisner R. 2008. The early detection of ovarian cancer: From traditional methods to proteomics. can we really do better than serum CA-125? *Am J Obstet Gynecol* 199(3):215-23.
- Obata K, Morland SJ, Watson RH, Hitchcock A, Chenevix-Trench G, Thomas EJ, Campbell IG. 1998. Frequent PTEN/MMAC mutations in endometrioid but not serous or mucinous epithelial ovarian tumors. *Cancer Res* 58(10):2095-7.
- Ota T, Gilks CB, Longacre T, Leung PC, Auersperg N. 2007. HOXA7 in epithelial ovarian cancer: Interrelationships between differentiation and clinical features. *Reprod Sci* 14(6):605-14.
- Ota T, Choi KB, Gilks CB, Leung PC, Auersperg N. 2006. Cell type- and stage-specific changes in HOXA7 protein expression in human ovarian folliculogenesis: Possible role of GDF-9. *Differentiation* 74(1):1-10.
- Ota T, Klausen C, Salamanca MC, Woo HL, Leung PC, Auersperg N. 2009. Expression and function of HOXA genes in normal and neoplastic ovarian epithelial cells. *Differentiation* 77(2):162-71.
- Palacios J and Gamallo C. 1998. Mutations in the beta-catenin gene (CTNNB1) in endometrioid ovarian carcinomas. *Cancer Res* 58(7):1344-7.
- Palma M, Ristori E, Ricevuto E, Giannini G, Gulino A. 2006. BRCA1 and BRCA2: The genetic testing and the current management options for mutation carriers. *Crit Rev Oncol Hematol* 57(1):1-23.

- Quirk SM, Cowan RG, Huber SH. 1997. Fas antigen-mediated apoptosis of ovarian surface epithelial cells. *Endocrinology* 138(11):4558-66.
- Raman V, Martensen SA, Reisman D, Evron E, Odenwald WF, Jaffee E, Marks J, Sukumar S. 2000. Compromised HOXA5 function can limit p53 expression in human breast tumours. *Nature* 405(6789):974-8.
- Roh MH, Kindelberger D, Crum CP. 2009. Serous tubal intraepithelial carcinoma and the dominant ovarian mass: Clues to serous tumor origin? *Am J Surg Pathol* 33(3): 376-83.
- Salamanca CM, Maines-Bandiera SL, Leung PC, Hu YL, Auersperg N. 2004. Effects of epidermal growth factor/hydrocortisone on the growth and differentiation of human ovarian surface epithelium. *J Soc Gynecol Investig* 11(4):241-51.
- Salazar H, Godwin AK, Daly MB, Laub PB, Hogan WM, Rosenblum N, Boente MP, Lynch HT, Hamilton TC. 1996. Microscopic benign and invasive malignant neoplasms and a cancer-prone phenotype in prophylactic oophorectomies. *J Natl Cancer Inst* 88(24):1810-20.
- Salvador S, Gilks B, Kobel M, Huntsman D, Rosen B, Miller D. 2009. The fallopian tube: Primary site of most pelvic high-grade serous carcinomas. *Int J Gynecol Cancer* 19(1):58-64.
- Satokata I, Benson G, Maas R. 1995. Sexually dimorphic sterility phenotypes in Hoxa10-deficient mice. *Nature* 374(6521):460-3.
- Sauvageau G, Lansdorp PM, Eaves CJ, Hogge DE, Dragowska WH, Reid DS, Largman C, Lawrence HJ, Humphries RK. 1994. Differential expression of homeobox genes in functionally distinct CD34+ subpopulations of human bone marrow cells. *Proc Natl Acad Sci U S A* 91(25):12223-7.
- Scott MP. 1992. Vertebrate homeobox gene nomenclature. *Cell* 71(4):551-3.
- Scully RE. 1995. Early de novo ovarian cancer and cancer developing in benign ovarian lesions. *Int J Gynaecol Obstet* 49 Suppl:S9-15.
- Seidman JD, Horkayne-Szakaly I, Haiba M, Boice CR, Kurman RJ, Ronnett BM. 2004. The histologic type and stage distribution of ovarian carcinomas of surface epithelial origin. *Int J Gynecol Pathol* 23(1):41-4.
- Shih I and Kurman RJ. 2004. Ovarian tumorigenesis: A proposed model based on morphological and molecular genetic analysis. *Am J Pathol* 164(5):1511-8.
- Singer G, Oldt R, 3rd, Cohen Y, Wang BG, Sidransky D, Kurman RJ, Shih I. 2003. Mutations in BRAF and KRAS characterize the development of low-grade ovarian serous carcinoma. *J Natl Cancer Inst* 95(6):484-6.
- Singer G, Stohr R, Cope L, Dehari R, Hartmann A, Cao DF, Wang TL, Kurman RJ, Shih I. 2005. Patterns of p53 mutations separate ovarian serous borderline tumors and low- and high-grade carcinomas and provide support for a new model of ovarian

- carcinogenesis: A mutational analysis with immunohistochemical correlation. *Am J Surg Pathol* 29(2):218-24.
- So CW, Karsunky H, Wong P, Weissman IL, Cleary ML. 2004. Leukemic transformation of hematopoietic progenitors by MLL-GAS7 in the absence of Hoxa7 or Hoxa9. *Blood* 103(8):3192-9.
- Stuart ET, Haffner R, Oren M, Gruss P. 1995. Loss of p53 function through PAX-mediated transcriptional repression. *EMBO J* 14(22):5638-45.
- Sueblinvong T and Carney ME. 2009. Current understanding of risk factors for ovarian cancer. *Curr Treat Options Oncol* 10(1-2):67-81.
- Sundfeldt K, Piontekewitz Y, Ivarsson K, Nilsson O, Hellberg P, Brannstrom M, Janson PO, Enerback S, Hedin L. 1997. E-cadherin expression in human epithelial ovarian cancer and normal ovary. *Int J Cancer* 74(3):275-80.
- Syed V, Ulinski G, Mok SC, Yiu GK, Ho SM. 2001. Expression of gonadotropin receptor and growth responses to key reproductive hormones in normal and malignant human ovarian surface epithelial cells. *Cancer Res* 61(18):6768-76.
- Tan OL and Fleming JS. 2004. Proliferating cell nuclear antigen immunoreactivity in the ovarian surface epithelium of mice of varying ages and total lifetime ovulation number following ovulation. *Biol Reprod* 71(5):1501-7.
- Taylor HS, Vanden Heuvel GB, Igarashi P. 1997. A conserved hox axis in the mouse and human female reproductive system: Late establishment and persistent adult expression of the hoxa cluster genes. *Biol Reprod* 57(6):1338-45.
- Taylor HS, Igarashi P, Olive DL, Arici A. 1999. Sex steroids mediate HOXA11 expression in the human peri-implantation endometrium. *J Clin Endocrinol Metab* 84(3):1129-35.
- Taylor HS, Arici A, Olive D, Igarashi P. 1998. HOXA10 is expressed in response to sex steroids at the time of implantation in the human endometrium. *J Clin Invest* 101(7):1379-84.
- Thorsteinsdottir U, Sauvageau G, Hough MR, Dragowska W, Lansdorp PM, Lawrence HJ, Largman C, Humphries RK. 1997. Overexpression of HOXA10 in murine hematopoietic cells perturbs both myeloid and lymphoid differentiation and leads to acute myeloid leukemia. *Mol Cell Biol* 17(1):495-505.
- Tok EC, Ertunc D, Tataroglu C, Yazici G, Kanat H, Dilek S. 2006. Clinicopathologic study of the putative precursor lesions of epithelial ovarian cancer in low-risk women. *Int J Gynecol Cancer* 16(2):501-6.
- Tonary AM, Macdonald EA, Faught W, Senterman MK, Vanderhyden BC. 2000. Lack of expression of c-KIT in ovarian cancers is associated with poor prognosis. *Int J Cancer* 89(3):242-50.

- Veatch AL, Carson LF, Ramakrishnan S. 1994. Differential expression of the cell-cell adhesion molecule E-cadherin in ascites and solid human ovarian tumor cells. *Int J Cancer* 58(3):393-9.
- Villaescusa JC, Verrotti AC, Ferretti E, Farookhi R, Blasi F. 2004. Expression of hox cofactor genes during mouse ovarian follicular development and oocyte maturation. *Gene* 330:1-7.
- Werness BA, Afify AM, Bielat KL, Eltabbakh GH, Piver MS, Paterson JM. 1999. Altered surface and cyst epithelium of ovaries removed prophylactically from women with a family history of ovarian cancer. *Hum Pathol* 30(2):151-7.
- Winter WE, 3rd, Maxwell GL, Tian C, Carlson JW, Ozols RF, Rose PG, Markman M, Armstrong DK, Muggia F, McGuire WP, et al. 2007. Prognostic factors for stage III epithelial ovarian cancer: A gynecologic oncology group study. *J Clin Oncol* 25(24):3621-7.
- Wong AS, Maines-Bandiera SL, Rosen B, Wheelock MJ, Johnson KR, Leung PC, Roskelley CD, Auersperg N. 1999. Constitutive and conditional cadherin expression in cultured human ovarian surface epithelium: Influence of family history of ovarian cancer. *Int J Cancer* 81(2):180-8.
- Woo MM, Gilks CB, Verhage HG, Longacre TA, Leung PC, Auersperg N. 2004. Oviductal glycoprotein, a new differentiation-based indicator present in early ovarian epithelial neoplasia and cortical inclusion cysts. *Gynecol Oncol* 93(2):315-9.
- Yamashita T, Tazawa S, Yawei Z, Katayama H, Kato Y, Nishiwaki K, Yokohama Y, Ishikawa M. 2006. Suppression of invasive characteristics by antisense introduction of overexpressed HOX genes in ovarian cancer cells. *Int J Oncol* 28(4):931-8.
- Yao DS, Li L, Garson K, Vanderhyden BC. 2006. The mouse ovarian surface epithelium cells (MOSE) transformation induced by c-myc/K-ras in. *Zhonghua Zhong Liu Za Zhi* 28(12):881-5.
- Yoshida H, Broaddus R, Cheng W, Xie S, Naora H. 2006. Deregulation of the HOXA10 homeobox gene in endometrial carcinoma: Role in epithelial-mesenchymal transition. *Cancer Res* 66(2):889-97.
- Zakany J and Duboule D. 2007. The role of hox genes during vertebrate limb development. *Curr Opin Genet Dev* 17(4):359-66.
- Zhang X, Zhu T, Chen Y, Mertani HC, Lee KO, Lobie PE. 2003. Human growth hormone-regulated HOXA1 is a human mammary epithelial oncogene. *J Biol Chem* 278(9):7580-90.
- Zhao Y, Yamashita T, Ishikawa M. 2005. Regulation of tumor invasion by HOXB13 gene overexpressed in human endometrial cancer. *Oncol Rep* 13(4):721-6.