THE ROLE OF LUTEAL PHASE FALLOPIAN TUBE EPITHELIUM IN HIGH-GRADE OVARIAN SEROUS CARCINOMA

BY

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A THESIS SUBMITTED IN CONFORMITY WITH THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY
DEPARTMENT OF LABORATORY MEDICINE AND PATHOBIOLOGY
UNIVERSITY OF TORONTO

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THE ROLE OF LUTEAL PHASE FALLOPIAN TUBE EPITHELIUM IN HIGH-GRADE OVARIAN SEROUS CARCINOMA

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ABSTRACT

Studies of prophylactic salpingectomy specimens from BRCA1/2 mutation carriers, at risk for tubal and ovarian high-grade serous carcinoma (SerCa), have consistently revealed occult carcinomas and putative histological cancer precursors in the distal fallopian tube epithelium (FTE), supporting the FTE as the source of SerCa. In this thesis I molecularly characterized and compared non-malignant FTE from mutation carriers (FTEb) and control patients (FTEn) to identify alterations that may predispose to malignant transformation. Gene expression profiling of laser capture microdissected FTEn, FTEb and SerCa indicated that SerCa have similar molecular profiles whether of presumed ovarian or tubal origin, supporting the notion they share a common cell of origin within the FTE. Furthermore, FTEb samples obtained during the post-ovulatory luteal phase showed gene expression profiles closely resembling SerCa samples, suggesting that the luteal phase milieu may contribute to serous carcinogenesis. An initial hypothesis was that FTEb may respond differently to luteal progesterone compared to FTEn, via differential expression of progesterone receptor (PR) isoforms. However, similar relative isoform expression in FTEn and FTEb samples suggested that a luteal phase-associated factor other than progesterone directs gene expression changes in FTEb. The possibility that FTEb respond differently to ovulation-associated inflammatory cytokines that are locally elevated during the luteal phase was next investigated. Importantly, FTEb specimens previously found to cluster with SerCa based on their global gene expression
profiles showed evidence of increased nuclear factor-κB (NFκB)-dependent (pro-inflammatory) signalling and diminished glucocorticoid receptor (GR)-dependent (anti-inflammatory) signalling. Furthermore, I demonstrate that disabled homolog 2 (DAB2), an adaptor molecule decreased in SerCa and FTE luteal samples, enhances both GR-mediated transactivation and suppression of NFκB signalling, implicating DAB2 as a crucial determinant of inflammatory signalling and ovarian cancer risk. Altogether, this thesis identifies gene expression changes in FTE from BRCA mutation carriers during the post-ovulatory luteal phase that parallel those detected in SerCa. The data support a proposed novel testable model for predisposing events contributing to SerCa that centres on an altered ability to quickly resolve the pro-inflammatory environment created by the ovulatory event.
ACKNOWLEDGEMENTS

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Thank you also to the members of the Brown lab (including Shadab, Alex, Prem, Taymaa and most recently Jim) for their constructive criticism during our intense lab meetings, and for putting up with me over the past year. Thanks to Carl Virtanen for consistently and patiently providing bioinformatics guidance, and to my floor mates (Sascha, Shakib, Shawn, Livia, Antonella and Domenica) for the many late-night chats and pub nights. Special thanks go out to Jocelyn, for her close friendship both at the lab and in the real world. I’d also like to thank my ‘non-science’ support system, most notably Mandy, Jay and Lindsay for our many emergency meetings, and Nicole and Shannon for their enduring friendship. Finally, I need to thank my family (mom, dad, Darrin and Darcia), with a special shout-out to dad for working so hard to perfect his ‘elevator speech.’
# CONTENTS

Abstract ii
Acknowledgements iv
Table of Contents v
Original Publications/Manuscripts x
List of Figures xi
List of Tables xiii
List of Abbreviations xiv
Permission Letters for Copyrighted Material xvii

## CHAPTER 1: LITERATURE REVIEW

1.1 Overview 1

1.2 Histological categorization of epithelial ovarian cancers 2

1.3 High-grade serous carcinoma pathway 4

1.4 Ovarian cancer risk factors: BRCA1/2 mutations 6
   1.4.1 Association of BRCA1/2 mutations with ovarian cancer risk 6
   1.4.2 BRCA1/2 and DNA repair 7
   1.4.3 BRCA1 and cell cycle regulation 8
   1.4.4 BRCA1 control of steroid hormone receptor activity 9
   1.4.5 Phenotypes associated with BRCA1/2 heterozygosity 10

1.5 Ovarian cancer risk factors: reproductive history and oral contraception 11
   1.5.1 Oral contraceptives and ovarian cancer risk 11
   1.5.2 Risks associated with parity and breast feeding 12
   1.5.3 Risk associated with lifetime number of ovulatory cycles 12

1.6 Ovarian cancer risk factors: hormone replacement therapy 13

1.7 Ovarian cancer risk factors: tubal ligation 14

1.8 Ovarian cancer etiology: Incessant Ovulation Hypothesis 15
   1.8.1 Brief overview of ovarian cycle 15
   1.8.2 Ovulation as an acute inflammatory response 18
   1.8.3 Incessant Ovulation Hypothesis 19

1.9 Ovarian cancer etiology: potential protective role of progesterone signalling 22
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.4</td>
<td>Results and Discussion</td>
<td>64</td>
</tr>
<tr>
<td>2.4.1</td>
<td>High-grade ovarian SerCa and fallopian tube SerCa exhibit</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>indistinguishable gene expression profiles</td>
<td></td>
</tr>
<tr>
<td>2.4.2</td>
<td>Differential expression of genes in a subset of histologically</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>normal FTEb samples</td>
<td></td>
</tr>
<tr>
<td>2.4.3</td>
<td>Differential effect of the ovarian cycle in BRCA1/2 mutation</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td>carriers</td>
<td></td>
</tr>
<tr>
<td>2.4.4</td>
<td>Identification of protein-protein interaction networks potentially</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>involved in initiation of SerCa</td>
<td></td>
</tr>
<tr>
<td>2.4.5</td>
<td>Differential expression of SKIL and DAB2 in FTEb samples</td>
<td>79</td>
</tr>
<tr>
<td></td>
<td>during the luteal phase</td>
<td></td>
</tr>
<tr>
<td></td>
<td>**CHAPTER 3: LOSS OF PROGESTERONE RECEPTOR ALPHA IN</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>LUTEAL PHASE FALLOPIAN TUBE EPITHELIUM</td>
<td></td>
</tr>
<tr>
<td>3.1</td>
<td>Abstract</td>
<td>85</td>
</tr>
<tr>
<td>3.2</td>
<td>Introduction</td>
<td>86</td>
</tr>
<tr>
<td>3.3</td>
<td>Materials and Methods</td>
<td>87</td>
</tr>
<tr>
<td>3.3.1</td>
<td>RT-qPCR for PR mRNA</td>
<td>87</td>
</tr>
<tr>
<td>3.3.2</td>
<td>PR isoform-specific IHC</td>
<td>87</td>
</tr>
<tr>
<td>3.4</td>
<td>Results</td>
<td>88</td>
</tr>
<tr>
<td>3.4.1</td>
<td>Decreased expression of PR mRNA in FTEb and SerCa</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>specimens</td>
<td></td>
</tr>
<tr>
<td>3.4.2</td>
<td>Increased expression of PRA- and PRB-influenced genes in</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>FTEb luteal and FTEb(S) samples</td>
<td></td>
</tr>
<tr>
<td>3.4.3</td>
<td>Decreased PRA protein expression in luteal and SerCa specimens</td>
<td>94</td>
</tr>
<tr>
<td>3.4.4</td>
<td>Decreased PRB protein expression in SerCa specimens</td>
<td>94</td>
</tr>
<tr>
<td>3.4.5</td>
<td>Decreased nuclear PRA:B ratio in luteal relative to follicular</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>samples</td>
<td></td>
</tr>
<tr>
<td>3.4.6</td>
<td>Decreased PRA nuclear intensity in luteal samples</td>
<td>101</td>
</tr>
<tr>
<td>3.4.7</td>
<td>Selective loss of cytoplasmic PRA in luteal samples</td>
<td>101</td>
</tr>
<tr>
<td>3.5</td>
<td>Discussion</td>
<td>104</td>
</tr>
<tr>
<td>4.1</td>
<td>Abstract</td>
<td>111</td>
</tr>
</tbody>
</table>

**CHAPTER 4: ALTERED GR AND NFκB INFLAMMATORY SIGNALLING**

**IN POST-OVULATORY FTEB(S) AND SERCA SAMPLES**
CHAPTER 5: DAB2 ACTIVATES GLUCOCORTICOID RECEPTOR-MEDIATED ANTI-INFLAMMATORY SIGNALLING IN OVARIAN CANCER CELLS

5.1 Abstract

5.2 Introduction

5.3 Materials and Methods

5.4 Results

5.5 Discussion
5.5 Discussion 162

CHAPTER 6: GENERAL DISCUSSION 168

6.1 An alternative model for serous carcinogenesis 175

CHAPTER 7: REFERENCES 182

APPENDICES

A1. Genes differentially expressed in subset of FTEb specimens 1A

A2. Genes differentially expressed between the luteal and follicular phase in mutation carriers 23A


III. Tone, A., Virtanen, C., Shaw, P.A., and Brown, T.J. Altered GR and NFκB inflammatory signalling in post-ovulatory FTEb(S) and SerCa samples. Manuscript in preparation.

# LIST OF FIGURES

| FIGURE 1.1: Breakdown of ovarian cancer types | 3 |
| FIGURE 1.2: Overview of the 28 day ovarian cycle | 17 |
| FIGURE 1.3: Hypothesis that ovarian cancer arises from inclusion cysts lined by OSE | 20 |
| FIGURE 1.4: Structural organization of human PRA and PRB protein isoforms | 25 |
| FIGURE 1.5: Schematic representation of canonical NFκB pathway | 34 |
| FIGURE 1.6: Schematic representation of anti-inflammatory signalling by GR | 37 |
| FIGURE 1.7: Segments of the human fallopian tube | 41 |
| FIGURE 1.8: FTE as the cell of origin of ‘ovarian’ SerCa | 47 |
| FIGURE 2.1: Comparison of fallopian tube and ovarian SerCas | 65 |
| FIGURE 2.2: Identification of differentially expressed genes in histologically normal FTEb | 69 |
| FIGURE 2.3: The effect of the ovarian cycle on FTE gene expression in BRCA1/2 mutation carriers and normal controls | 72 |
| FIGURE 2.4: Protein-protein interaction subnetwork potentially involved in initiation of SerCa | 77 |
| FIGURE 2.5: Expression of SKIL, DAB2, and cdc2 in FTEb luteal and SerCa samples | 80 |
| FIGURE 3.1: Decreased expression of PR mRNA in FTEb and SerCa specimens | 89 |
| FIGURE 3.2: Decreased PRA protein expression in FTE luteal and SerCa specimens | 95 |
| FIGURE 3.3: Decreased PRB protein expression in SerCa specimens | 97 |
| FIGURE 3.4: Decreased nuclear PRA:B in luteal samples | 100 |
| FIGURE 3.5: Decreased PRA nuclear intensity in FTE luteal samples | 102 |
FIGURE 3.6: Selective loss of cytoplasmic PRA in FTE luteal samples 103

FIGURE 4.1: Increased expression of GR mRNA in FTEb(S) samples 115

FIGURE 4.2: Increased expression of nuclear GR protein in FTEb(S) samples 117

FIGURE 4.3: Increased expression of RelA mRNA in FTEb(S) samples 125

FIGURE 4.4: Increased expression of nuclear and cytoplasmic RelA protein in FTEb(S) samples 126

FIGURE 4.5: Altered expression of inflammatory mediators in SerCa and select FTE 129

FIGURE 5.1: ES2 ovarian cancer cells have functional GR signalling 150

FIGURE 5.2: DAB2 enhances GR transactivation activity 153

FIGURE 5.3: DAB2 enhances GR-mediated repression of NFκB activity 155

FIGURE 5.4: DAB2 alters the expression of select NFκB target genes 156

FIGURE 5.5: DAB2 does not alter RelA mRNA expression 160

FIGURE 5.6: DAB2 interacts with GR and RelA proteins in vehicle- and dex-treated cells 161

FIGURE 6.1: Proposed model for serous carcinogenesis incorporating a central role for DAB2 176

FIGURE 6.2: Risk of SerCa is determined by the balance of pro- and anti-inflammatory signalling 181
LIST OF TABLES

TABLE 2.1: Relevant clinical data for samples used for gene expression profiling 58

TABLE 3.1: PRB-upregulated genes showing increased expression in FTEb luteal + FTEb(S) samples 92

TABLE 3.2: PRA-upregulated genes showing increased expression in FTEb luteal + FTEb(S) samples 93

TABLE 4.1: Functional categories of altered GR-induced genes 119

TABLE 4.2: Genes upregulated by GR showing decreased expression in FTEb(S) samples 120

TABLE 4.3: Functional categories of altered GR-repressed genes 121

TABLE 4.4: Genes downregulated by GR showing increased expression in FTEb(S) samples 123

TABLE 4.5: Genes upregulated by NFκB showing increased expression in FTEb(S) samples 131
### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>11βHSD type 1</td>
<td>11β-hydroxysteroid dehydrogenase type 1</td>
</tr>
<tr>
<td>ACTB</td>
<td>b-actin</td>
</tr>
<tr>
<td>ACTH</td>
<td>adrenocorticotropic hormone</td>
</tr>
<tr>
<td>AIB1</td>
<td>amplified in breast cancer 1</td>
</tr>
<tr>
<td>AP-1</td>
<td>activator protein-1</td>
</tr>
<tr>
<td>AR</td>
<td>androgen receptor</td>
</tr>
<tr>
<td>BARΔ1</td>
<td>BRCA1 associated RING domain 1</td>
</tr>
<tr>
<td>BRCA1/2</td>
<td>breast cancer associated 1/2</td>
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<tr>
<td>BSO</td>
<td>bilateral salpingo-oophorectomy</td>
</tr>
<tr>
<td>BTVSQ</td>
<td>binary tree-structured vector quantization</td>
</tr>
<tr>
<td>CBP</td>
<td>CREB-binding protein</td>
</tr>
<tr>
<td>CCL2</td>
<td>chemokine (C-C motif) ligand 2 (also see MCP1)</td>
</tr>
<tr>
<td>CCNE</td>
<td>cyclin E</td>
</tr>
<tr>
<td>CDC2</td>
<td>cell division cycle 2</td>
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<tr>
<td>CDK2</td>
<td>cyclin-dependent kinase 2</td>
</tr>
<tr>
<td>CDKN2A</td>
<td>cyclin-dependent kinase inhibitor 2A</td>
</tr>
<tr>
<td>CHK1</td>
<td>checkpoint kinase 1</td>
</tr>
<tr>
<td>CI</td>
<td>confidence interval</td>
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<tr>
<td>COX2</td>
<td>cyclooxygenase 2</td>
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<tr>
<td>CRH</td>
<td>corticotrophin-releasing hormone</td>
</tr>
<tr>
<td>CTSB</td>
<td>cathepsin B</td>
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<td>DAB2</td>
<td>disabled homolog 2 mitogen-responsive phosphoprotein</td>
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<tr>
<td>DAB2si</td>
<td>DAB2-specific siRNA</td>
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<tr>
<td>DDB</td>
<td>DNA binding domain</td>
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<tr>
<td>Dex</td>
<td>dexamethasone</td>
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<tr>
<td>DIM</td>
<td>dimerization</td>
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<tr>
<td>DSB</td>
<td>double-stranded DNA break</td>
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<td>DUSP1</td>
<td>dual specificity phosphatase 1</td>
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<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
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<tr>
<td>e-NOS</td>
<td>endothelial nitric oxide synthase</td>
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<td>EOC</td>
<td>epithelial ovarian cancer</td>
</tr>
<tr>
<td>ER</td>
<td>estrogen receptor</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<tr>
<td>FDR</td>
<td>false discovery rate</td>
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<tr>
<td>FORKO</td>
<td>FSH receptor knockout</td>
</tr>
<tr>
<td>FSH</td>
<td>follicle-stimulating hormone</td>
</tr>
<tr>
<td>FTE</td>
<td>fallopian tube epithelium</td>
</tr>
<tr>
<td>FTEb</td>
<td>non-malignant FTE from BRCA1/2 mutation carrier</td>
</tr>
<tr>
<td>FTEb(N)</td>
<td>FTEb clustering with FTEn (Chapter 2)</td>
</tr>
<tr>
<td>FTEb(S)</td>
<td>FTEb clustering with SerCa (Chapter 2)</td>
</tr>
<tr>
<td>FTE_con</td>
<td>distal FTE contralateral to ovulating ovary</td>
</tr>
<tr>
<td>FTE_ips</td>
<td>distal FTE ipsilateral to ovulating ovary</td>
</tr>
<tr>
<td>FTEn</td>
<td>non-malignant FTE from control patient</td>
</tr>
<tr>
<td>GILZ</td>
<td>glucocorticoid inducible leucine zipper</td>
</tr>
<tr>
<td>GnRH</td>
<td>gonadotropin releasing hormone</td>
</tr>
<tr>
<td>GR</td>
<td>glucocorticoid receptor</td>
</tr>
<tr>
<td>Grb2</td>
<td>growth factor receptor binding protein 2</td>
</tr>
<tr>
<td>GRE</td>
<td>glucocorticoid response element</td>
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</tbody>
</table>
GRIP1  glucocorticoid receptor-interacting protein 1
GSK3β  glycogen synthase kinase 3 beta
HOX  homeobox genes
HPA  hypothalamic-pituitary-adrenal axis
HR  homologous recombination
HRT  hormone replacement therapy
HSP90  heat shock protein 90
I2D  Interlogous Interaction Database
ICAM1  intercellular adhesion molecule 1
ID  inhibitory domain
IGFBP1  insulin-like growth factor binding protein 1
IHC  immunohistochemistry
IKK  IκB-kinase complex
IL-1  interleukin-1
IL-8  interleukin-8
i-NOS  inducible nitric oxide synthase
LBD  ligand binding domain
LCM  laser capture microdissection
LGSC  low-grade serous carcinoma
LH  luteinizing hormone
LOH  loss of heterozygosity
MCP1  monocyte chemoattractant peptide 1 (also see CCL2)
MEC  mammary epithelial cells
MMTV  mouse mammary tumor virus
MSK-1  mitogen- and stress-activated protein kinase-1
NAVigAtOR  Network Analysis, Visualization, and Graphing TORonto
NFκB  nuclear factor-κB
NHEJ  non-homologous end-joining
nPR  nuclear PR
NR3C1  nuclear receptor subfamily 3, group C, member 1
NSAIDS  nonsteroidal anti-inflammatory drugs
NTsi  non-targeting siRNA
OCP  oral contraceptive pill
OGP  oviductal glycoprotein
OSE  ovarian surface epithelium
PCOS  polycystic ovary syndrome
PGE2  prostaglandin E2
PID  pelvic inflammatory disease
PKA  protein kinase A
PKAc  catalytic subunit of PKA
PKC  protein kinase C
PP2B  protein phosphatase 2B
PR  progesterone receptor
PRE  progesterone response element
RB  retinoblastoma protein
RelA  v-rel reticuloendotheliosis viral oncogene homolog A
RMA  robust multiarray average
RR  relative risk
RT-qPCR  real-time quantitative PCR
SAM  Significance Analysis of Microarrays
SerCa  serous carcinoma
SKIL  Ski-like
SOD2  superoxide dismutase 2 mitochondrial
<table>
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<tr>
<td>SRC1</td>
<td>steroid receptor coactivator 1</td>
</tr>
<tr>
<td>SRC3</td>
<td>steroid receptor coactivator 3</td>
</tr>
<tr>
<td>TGFβ</td>
<td>transforming growth factor beta</td>
</tr>
<tr>
<td>TIC</td>
<td>tubal intraepithelial carcinoma</td>
</tr>
<tr>
<td>TNFα</td>
<td>tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TNFRSF6</td>
<td>tumor necrosis factor receptor superfamily member 6</td>
</tr>
<tr>
<td>VCAM1</td>
<td>vascular cell adhesion molecule 1</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>WT1</td>
<td>Wilms tumor 1</td>
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</tbody>
</table>
FIGURE 1.3

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CHAPTER 2

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CHAPTER 1
LITERATURE REVIEW

1.1. Overview

Epithelial ovarian cancer (EOC) is the fifth leading cause of cancer-related death in Canadian women and the most lethal gynaecological malignancy. Ovarian cancer is associated with an exceedingly high mortality rate (>70%), with an estimated 2400 women being newly diagnosed and 1700 women dying of the disease in Canada each year (1). This statistic has remained relatively unchanged for the past 20 years, despite advances in treatment. A major contributor to the poor prognosis of ovarian cancer is the fact that >75% of patients present with advanced stage extra-ovarian disease due to the lack of reliable early detection markers. While most of these tumours initially respond to taxol and platinum-based combination chemotherapy, disease usually recurs in a resistant form within 2 years, resulting in an overall 5-year survival rate of <30%. In contrast, cancers diagnosed at an early stage (when disease is confined to the ovary) can be cured with surgery alone, with an overall 5-year survival rate approaching 90% (2-4). Because of this discrepancy, the focus of much research is aimed at improving the early detection of ovarian cancer, through better understanding of its underlying biology.

Ovarian epithelial tumours are thought to arise from the ovarian surface epithelium (OSE), a modified mesothelial layer with both mesenchymal and epithelial characteristics (2, 5). Several theories of how OSE cells may undergo malignant transformation have been put forth, largely guided by epidemiologic studies of ovarian cancer risk factors mostly associated with a woman’s reproductive history. These will be outlined in the following sections, and have included hypotheses concerning repeated cycles of ovulation-induced trauma and repair, exposure to reproductive hormones (e.g. estrogen, progesterone, androgen and gonadotropins) and ovarian inflammation. Despite these theories for ovarian cancer
development, precursor lesions for high-grade serous carcinoma (SerCa), the most common and lethal histological type of EOC, have not been identified in OSE cells (the presumed ovarian cell of origin). Recently, unexpected findings of occult SerCa in prophylactically removed fallopian tubes of BRCA1/2 mutation carriers, at elevated risk for ovarian and fallopian tube SerCa, have led to the description of putative SerCa precursors in the distal fallopian tube epithelium (FTE). These discoveries have led investigators to postulate that ‘ovarian’ SerCa is not derived from the OSE, but rather the FTE.

The focus of this thesis was to molecularly characterize and compare non-malignant FTE from BRCA1/2 mutation carriers (FTEb) and control patients (FTEn) to identify alterations involved in predisposition to SerCa. The unexpected finding that FTEb obtained during the luteal phase of the ovarian cycle most closely resembles SerCa led us to investigate whether key pathways typically active during the luteal phase (e.g. progesterone, glucocorticoid and/or inflammatory signalling) are altered in these specimens and may therefore contribute to the observed gene expression changes.

1.2. Histological categorization of epithelial ovarian cancers

Unlike the relatively uncommitted phenotype of the OSE, the four main histological subtypes of EOC (serous, endometrioid, clear cell and mucinous) resemble the epithelia of the Müllerian duct (fallopian tube, uterine endometrium and endocervix respectively) (Figure 1.1). Although EOC has historically been considered a single disease, each histotype is characterized by distinct molecular abnormalities, predisposing risk factors and clinical behaviour (e.g. stage at presentation, response to chemotherapy and survival), strongly suggesting varying etiologies (4).

Mucinous carcinomas, which account for <5% of EOC, present at an early stage and frequently show transition from a borderline to malignant tumour. Clear cell carcinomas constitute another 5% of EOC, frequently present at stage I/II and are associated with
FIGURE 1.1: Breakdown of ovarian cancer types. A small proportion of ovarian cancers arise from either ovarian stromal or germ cells. The remainder (90%) are thought to arise from the OSE. Unlike the uncommitted phenotype of the OSE, the four main histological subtypes of EOC resemble the epithelia of the Müllerian duct (indicated in brackets). The most common (80-85%) EOC subtype is serous carcinoma, which resembles the secretory epithelium of the fallopian tube.
endometriosis in >50% of cases. Endometrioid carcinomas (10% of EOC) account for 50% of all stage I tumours, are associated with endometriosis and frequently show abnormalities in β-catenin, the major component of the canonical Wnt signalling pathway. The most common (80-85%) EOC subtype is serous carcinoma. In contrast to the other histotypes, serous carcinoma is typically high-grade and presents at an advanced stage of disease >90% of the time and therefore represents the major clinical challenge of EOC (3, 4, 6-9).

Several studies have highlighted the differences between EOC subtypes (10-13). For instance, an epidemiologic study stratified by histotype reported a lack of association between reproductive factors (including parity and oral contraceptive (OCP) use) and risk of mucinous tumours, despite a protective effect in other subtypes (10). A retrospective comparative study of endometrioid and serous ovarian cancers revealed differences in patient age and disease stage at presentation, grade of disease, response to chemotherapy and overall- and progression-free survival (12). In addition, a recent study of 500 ovarian carcinomas by Koebel et al found that 19/21 tissue-based biomarkers were differentially expressed according to histotype rather than disease stage. Furthermore, some prognostic associations were inverse within the entire cohort vs. a particular subtype (e.g. Wilms tumor 1 (WT1) was an unfavourable prognostic marker within the entire cohort but favourable within high-grade serous tumours) (13).

1.3. High-grade serous carcinoma pathway

While the majority of serous tumours are high-grade, a small proportion are well-differentiated (low-grade). Clinical and morphologic observations, combined with recent genetic studies, suggest that low-grade serous carcinoma (LGSC) and high-grade (also referred to as ‘conventional’) serous carcinoma (SerCa) represent different pathologic entities with distinct pathways of development (and potentially unique cells of origin). LGSCs pursue an indolent course and progress gradually through a series of histological lesions over a period of up to 20
years. In contrast, SerCas appear to develop de novo in the absence of a distinct morphologic precursor, present at an advanced stage and are highly aggressive (3, 4, 14-17). Some authors have suggested that the intermediate steps of SerCa development are likely obscured due to the high rate of cellular proliferation (as shown by Ki-67 nuclear labelling) and subsequent rapid evolution of these tumours (14, 15). SerCa metastasizes early in its course, and is invariably found on the surface of the ovary, with frequent involvement of the fallopian tubes and peritoneal cavity (14, 17).

SerCa is characterized by unique molecular abnormalities, most notably somatic mutations in the tumour suppressor TP53 (up to 80% of cases) (9, 14, 15, 17, 18). The majority of these are missense mutations, leading to intranuclear accumulation of mutant p53 protein detectable by immunohistochemistry (IHC) (9, 18). The TP53 gene (and corresponding protein) is a critical determinant of cell fate that is frequently altered in human cancer, as it coordinates cell cycle arrest and apoptosis pathways following cellular stress (19, 20). Several studies have observed frequent alterations in cell cycle-associated proteins in SerCa, including but not limited to cyclin-dependent kinase inhibitor 2A (CDKN2A, also known as p16), retinoblastoma protein (RB) and cyclin E (CCNE) (9).

Approximately 15% of SerCa is associated with an underlying germline mutation in breast cancer associated 1/2 (BRCA1/2) (21-25). Although tumours associated with a BRCA1 mutation are diagnosed at a younger age than sporadic SerCa (25-27), these cancers are otherwise clinically and pathologically indistinguishable, suggesting a shared pathogenesis (14). Accordingly, sporadic ovarian cancers segregate into “BRCA1-like” and “BRCA2-like” tumours by gene expression profiling (28). Decreased nuclear expression of BRCA1 protein (via promoter hypermethylation) has also been observed in up to 80% of sporadic SerCa, suggesting that BRCA1 dysfunction may be commonly involved (26, 29, 30). In addition, BRCA1/2-associated and sporadic SerCa show a similar frequency of p53 alterations (25, 27, 31).
1.4. Ovarian cancer risk factors: BRCA1/2 mutations

1.4.1. Association of BRCA1/2 mutations with ovarian cancer risk

Currently, a family history and/or BRCA1/2 mutation are the most reliable predictors of future ovarian cancer development. The BRCA1 and BRCA2 genes are located on chromosomes 17q21 and 13q12-13 respectively, and each encode for large (predominantly) nuclear phosphoproteins with multiple functional domains (26, 32). Approximately 1 in 280 women carry a germline mutation in BRCA1 or BRCA2 (33). While the lifetime risk for developing ovarian cancer is 1.6% in the general population, this increases to up to 60% and up to 30% for BRCA1 or BRCA2 mutation carriers respectively (26, 33-35). A greater proportion of hereditary ovarian cancers occur in association with a BRCA1 (60%) vs. BRCA2 (30%) mutation, and BRCA1-associated cancers typically occur at a younger age (mean age late 40s/early 50s vs. 60 years for BRCA2 carriers) (33, 35, 36). Inheritance of a mutant BRCA1/2 allele follows an autosomal dominant pattern, followed by loss of the wild type allele leading to complete inactivation of the gene (observed as loss of heterozygosity (LOH) in tumour DNA) (20, 37).

More than 1000 distinct germline BRCA1/2 mutations have been identified; these are distributed throughout the coding regions of both genes and are primarily frameshift or nonsense mutations leading to creation of a premature stop codon and production of a truncated protein (26, 33, 35, 38-40). According to the Breast Cancer Information Core (41), the most commonly reported BRCA1 mutations are 185delAG (16.5%) and 5382insC (8.8%), and the most common BRCA2 mutation is 6174delT (9.6%). These same three mutations account for 98-99% of mutations identified in the Ashkenazi Jewish population (39, 40). Additional founder mutations have been identified in other populations and are reviewed in detail by Ramus et al (40). Furthermore, inheritance of specific mutations is associated with different levels of cancer risk; risk appears to depend in part on the location of the mutation and corresponding differences in the biology of the resulting protein (40).
1.4.2. *BRCA1/2 and DNA repair*

Cells and tumours deficient for BRCA display severe genomic instability, characterized by aneuploidy, centrosomal amplification and chromosomal aberrations (32, 42), suggesting that intact BRCA1/2 proteins prevent malignant transformation by maintenance of genomic integrity (39, 43, 44). One way in which BRCA1 and BRCA2 preserve the integrity of the genome is through roles in DNA repair pathways. Both proteins facilitate the repair of double-stranded DNA breaks (DSBs) induced by genotoxic agents or replication errors, primarily by (error-free) homologous recombination (HR) in collaboration with RAD51 (19, 26, 37, 39). BRCA1 also interacts with the RAD50-MRE11-NBS protein complex, suggesting an additional role in repair by (error-prone) non-homologous end-joining (NHEJ) (19, 45). Accordingly, cancer-associated missense and truncating mutations of *BRCA1* lead to severely impaired fidelity of both HR and NHEJ (45, 46). Interestingly, BRCA1 has also been shown to possess E3 ubiquitin ligase activity at its N-terminus, and subsequently mediates E2-dependent ubiquitination *in vitro* via cooperation with BRCA1 associated RING domain 1 (BARD1) (39, 46). Targets for BRCA1-dependent ubiquitination identified from *in vitro* studies have included BRCA1 itself, p53 and histone H2A (46). Ubiquitination of the H2A minor variant H2AX by BRCA1 has been suggested to modulate the response to DNA damage. Importantly, loss of BRCA1 repair function activates a p53-dependent response, resulting in cell cycle arrest and/or apoptosis to prevent the propagation of DNA damage. However, if p53 (or its target gene p21) is also inactivated, proliferation can continue despite the presence of unrepaired damage, leading to progressive accumulation of genomic errors and increased risk for malignant transformation (19, 20, 26, 32, 35, 37, 42, 47). Therefore, inactivation of the p53 pathway is likely necessary (but not sufficient) for malignant transformation of *BRCA1/2*-deficient cells, consistent with the high frequency of *TP53* mutations observed in *BRCA*-associated ovarian cancer. Notably, BRCA1 and p53 have also been found to collaborate extensively in normal cells. BRCA1 interacts with p53 and activates transcription of p53-regulated genes (19, 38, 48,
and may also act as a molecular scaffold for proteins involved in the fine-tuning of the p53-dependent damage response (19).

1.4.3. **BRCA1 and cell cycle regulation**

Cell cycle checkpoints act in concert with repair pathways to prevent the propagation of DNA damage and subsequent genomic instability. Importantly, BRCA1 protein has been shown to regulate progression through the cell cycle, with key roles in the G1/S, G2/M and spindle checkpoints (42). Transcription of **BRCA1** is generally induced in late G1, with levels peaking after the G1/S checkpoint. Hyperphosphorylation of BRCA1 protein is observed during the late G1 and S phases, with transient dephosphorylation following mitosis (42). Overexpression of wild-type but not tumour-derived mutants of BRCA1 has been found to induce G1 cell cycle arrest via transcriptional activation of the cyclin-dependent kinase inhibitor p21 (38). Ectopic expression of BRCA1 also results in dephosphorylation/activation of the critical G1/S regulator RB, potentially through interaction of BRCA1 and RB proteins, transactivation of p27 and/or inhibition of cyclin-dependent kinase 2 (CDK2) activity (38, 42, 48). BRCA1 also regulates the expression and/or phosphorylation of key effectors of the G2/M checkpoint, most notably checkpoint kinase 1 (CHK1), Wee1 kinase and 14-3-3 proteins (42). Consistently, expression of BRCA1 has been found to reduce levels of cyclin B1, a kinase directly inhibited by Wee1 (48). This checkpoint is crucial, as it ensures that cells with damaged DNA do not proceed into the mitosis phase where there would be an increased likelihood of abnormal chromosomes being passed to daughter cells (42). Finally, BRCA1 mutant (**BRCA1**<sup>11/11</sup>) cells show altered expression of several genes involved in the spindle checkpoint, which acts to ensure the accuracy of chromosome segregation during mitosis, thereby preventing aneuploidy (42). It is important to note that transcriptional regulation of repair and cell cycle genes by BRCA1 is not mediated by direct DNA binding, but rather interactions with components of the basal transcriptional machinery and coregulatory proteins (32).
1.4.4. **BRCA1 control of steroid hormone receptor activity**

Although roles in DNA repair and cell cycle regulation are consistent with a tumour suppressive function of BRCA1, they do not explain the specific occurrence of BRCA1-associated tumours in hormonally-responsive tissues such as the breast, ovary and fallopian tube. A potential explanation for this is provided by observations that BRCA1 protein can directly bind to and modulate the activity of steroid hormone receptors. For example, wild-type BRCA1 has been found to inhibit estrogen receptor (ERα)-dependent transactivation activity in a ligand-independent manner, via direct interaction of BRCA1 and ERα proteins and downregulation of the coregulatory protein p300 (32, 50-53). Consistently, BRCA1-deficient ovarian cancer cells and BRCA1-null mouse embryo fibroblasts demonstrate significantly increased ligand-independent ERα transcriptional activity compared to BRCA1-proficient cells. While expression of wild-type BRCA1 in BRCA1-deficient cells restored ligand-independent inhibition of ERα, cancer-associated BRCA1 mutants failed to do so (51). In addition, knockdown of BRCA1 has been shown to enhance stimulation of ERα activity with the largest difference observed at low doses of estradiol (32). Altogether, these findings suggest that loss of functional BRCA1 may lead to estrogen-independent activation of cell proliferation.

Wild-type BRCA1 has also been found to inhibit progesterone receptor (PR) transactivation activity and endogenous target gene expression, while knockdown of endogenous BRCA1 enhances ligand-dependent PR activity (53). Importantly, BRCA1 has been found to inhibit both isoforms of PR (PRA + PRB, see Section 1.9.2), and interacts with both proteins in vivo in a ligand-dependent manner. In vitro studies have revealed that sites within both the N-terminus and C-terminus of BRCA1 interact with a region shared between PRA and PRB (amino acids 166-687). A well-expressed but functionally defective BRCA1 protein harbouring a breast cancer-associated point mutation failed to block PR-dependent signalling (53).

In contrast to ER and PR, BRCA1 protein has been found to stimulate ligand-dependent androgen receptor (AR) transactivation activity in breast and prostate cancer cells, through
direct interaction of BRCA1 with AR and glucocorticoid receptor-interacting protein 1 (GRIP1) (32, 54). Cotransfection with any of the p160 coactivators GRIP1, steroid receptor coactivator 1 (SRC1) or amplified in breast cancer 1 (AIB1) synergistically enhanced BRCA1-dependent activation of AR (54). BRCA1 also enhanced AR-dependent p21 gene expression, as well as dihydrotestosterone-induced apoptosis of prostate cancer cells (32). Interestingly, wild-type (but not cancer-associated truncated) BRCA2 was similarly found to interact and synergize with GRIP1 to enhance AR-dependent transactivation activity, and was further found to cooperate with BRCA1 (55).

1.4.5. Phenotypes associated with BRCA1/2 heterozygosity

As previously discussed (Section 1.4.1), ovarian cancer tissue specimens obtained from BRCA mutation carriers show somatic LOH for the wild-type allele. Several studies have therefore attempted to address the questions of how and at which point during cancer development the wild-type BRCA1 or BRCA2 allele is lost, and whether the presence of only one wild-type allele (heterozygosity) is sufficient to maintain normal BRCA1/2 function. Phenotypic effects of BRCA heterozygosity that have been described include spontaneous recombination events and reduced efficiency of DNA DSB repair by HR, leading to an increased risk of cancer-promoting mutations (‘mutator phenotype’) (56). In addition, primary cultures derived from non-malignant mammary epithelial cells (MEC) of BRCA1 mutation carriers showed altered differentiation properties and increased cell proliferation and clonal growth, prior to LOH. Furthermore, gene expression profiles of colonies from BRCA1 heterozygotes anticipated those found in BRCA1-associated breast cancer (57). Expression of PR was also found to be increased in the normal breast epithelium adjacent to invasive carcinoma in BRCA1-associated (compared to sporadic) breast cancer. Importantly, the wild-type BRCA1 allele was retained in the normal epithelium in all tested cases, suggesting that partial loss of BRCA-dependent function leads to deregulation of PR expression, likely through
derepression of ER activity (58). One study also reported that lymphocytes containing a heterozygous \( BRCA1/2 \) mutation are more radiosensitive than lymphocytes obtained from women with no history of cancer (59). The concept of BRCA haploinsufficiency has also been supported in animal models, as \( Brca1^{+/−} \) mice exposed to ionizing radiation have a 3-5-fold higher incidence of ovarian tumours compared to \( Brca1^{+/+} \) mice, with retention of the wild-type allele in the tumours from heterozygous mice (60).

1.5. Ovarian cancer risk factors: reproductive history and oral contraception

1.5.1. Oral contraceptives and ovarian cancer risk

In addition to highly penetrant mutations in \( BRCA1/2 \), epidemiologic studies have determined additional (low-penetrance) modifiers of ovarian cancer risk, mostly associated with a woman’s reproductive history. Multiple studies have reported a substantial protective effect for use of OCPs in the general population, with greater risk reduction observed with increasing duration of use (61). For instance, a pooled analysis of six population-based case-control studies found a 34% risk reduction for women who have used OCPs, and a 70% risk reduction for greater than 5 years of use (62). The risk reduction associated with OCP use has been reported to persist for 10-30 years after cessation of use (63, 64). Notably, a case-control study stratifying by histological type observed a strong protective effect for ever use of OCPs within serous carcinomas specifically [relative risk (RR)= 0.56, 95% confidence interval (CI): 0.42-0.74] (65). Importantly, OCP use has similarly been associated with reduced risk among \( BRCA \) mutation carriers (62, 66-68). For instance, a recent matched case-control study by McLaughlin et al (consisting of 799 documented \( BRCA1/2 \) mutation carriers with a history of invasive cancer and 2424 carriers without ovarian cancer) reported a reduced risk for ever use of OCPs among both \( BRCA1 \) (odds ratio= 0.56, CI: 0.45-0.71; \( p<0.0001 \)) and \( BRCA2 \) (odds ratio= 0.39, CI: 0.23-0.66; \( p=0.0004 \)) mutation carriers. There was also a significant trend for increasing protection with increasing duration of OCP use (\( p<0.0001 \)) (66).
1.5.2. Risks associated with parity and breast feeding

Parity and breastfeeding have also consistently been associated with ovarian cancer risk reduction in the general population and BRCA1/2 mutation carriers, with greater protection provided by an increased number of full-term pregnancies and longer duration of total lactation time (61, 62, 66, 69). Interestingly, in the study by McLaughlin et al, parity was associated with reduced risk for women with a documented BRCA1 mutation (odds ratio= 0.67, CI: 0.46-0.96; p=0.03), while it increased risk among BRCA2 mutation carriers (odds ratio= 2.74, CI: 1.18-6.41; p=0.02) (66). Within BRCA1 mutation carriers, the odds ratio for each successive birth was 0.87 (CI: 0.79-0.95, p=0.003), similar to that observed by a separate group (70). Several studies have also found a trend of decreasing risk with increasing age at first birth (63). With respect to breastfeeding, McLaughlin et al observed a similar reduction in risk for BRCA1 (odds ratio= 0.74, CI: 0.56-0.97; p=0.03) and BRCA2 (odds ratio= 0.72, CI: 0.41–1.29; p=0.27) mutation carriers, although the latter association did not reach statistical significance.

Combined protective effects for parity and breastfeeding could also be observed in BRCA1 mutation carriers, with an odds ratio of 0.43 (CI: 0.3-0.62) observed for parous women who had breastfed for >1 year compared with nulliparous women (p<0.0001) (66).

1.5.3. Risk associated with lifetime number of ovulatory cycles

It is important to note that OCP use, parity and breastfeeding (69) are each associated with suppression of ovulation. Consistently, a population-based case-control study (consisting of 558 EOC cases and 607 controls) found a positive association between lifetime ovulatory (log)years and risk of ovarian cancer, specifically among premenopausal women (odds ratio= 2.49, CI: 1.53-4.05) (64). The number of lifetime ovulations was determined by subtracting the total years of anovulatory periods (due to pregnancy, breast feeding, OCP use and amenorrhea) from total menstrual years, multiplied by the estimated number of cycles per year. Interestingly, the association of lifetime ovulations with risk was not observed for
postmenopausal women (odds ratio=0.88, CI: 0.49-1.58), suggesting that number of ovulatory events is only involved in premenopausal ovarian cancer development. Consistent with the findings above, individual factors associated with anovulation (OCPs, pregnancy and breastfeeding) were each associated with reduced risk in both pre- and postmenopausal women. While the extent of protection provided by breastfeeding and pregnancy were similar among both pre- and postmenopausal women, there was a trend for greater protection with prolonged OCP use (equal to/greater than 5.4 years vs. never use) in premenopausal (odds ratio=0.28, CI: 0.15-0.52) vs. postmenopausal (odds ratio=0.58, CI: 0.31-1.08) women (p for interaction=0.20) (64).

1.6. Ovarian cancer risk factors: hormone replacement therapy

Reports on the association of postmenopausal hormone replacement therapy (HRT) and ovarian cancer risk have been inconclusive, with some cohort and case-control studies showing a modest increase in risk for long-term HRT (RR around 1.5) and others showing no effect (61, 63, 71, 72). The largest study to date, the UK Million Women Study (consisting of 948,576 postmenopausal women), found a modest yet significantly increased risk for current users of HRT (RR= 1.20, CI: 1.09-1.32; p=0.0002) compared to those women who had never used HRT. Risk for current HRT users was highest for serous tumours (RR= 1.53, CI: 1.31-1.79; p<0.0001) and increased according to duration of use. Overall, HRT was associated with one extra ovarian cancer in roughly 2500 current (vs. never) users, and one extra ovarian cancer-related death in 3300 current users. Importantly, past users of HRT were not at an increased risk for ovarian cancer (RR= 0.98, CI: 0.88-1.11). Also, while unopposed estrogen formulations were associated with higher risk compared to combination (estrogen + progesterone) HRT, this did not reach statistical significance (73). Interestingly, the Women’s Health Initiative randomized trial also showed an increased risk for women assigned to combination HRT compared to placebo (RR= 1.58, CI: 0.77-3.24) (63). Finally, a matched
case-control study found no association of (ever use of) HRT with ovarian cancer incidence in \textit{BRCA1/2} mutation carriers (odds ratio=0.93, CI: 0.56-1.56). A lack of association was also found when subjects were stratified by mutation status or duration of HRT use was considered (74), although this may be partly due to the small numbers (162 matched pairs) and the high baseline risk in mutation carriers.

1.7. Ovarian cancer risk factors: tubal ligation

Several studies have reported an inverse association of sterilization by tubal ligation and ovarian cancer risk in the general population, with evidence that protection continues >20 years following the procedure (62, 67, 75-78). Of particular note, the Nurses’ Health Study (consisting of >100,000 women) reported a substantial reduced risk for women who had undergone tubal ligation (odds ratio= 0.33, CI: 0.16-0.64) after 12 years of follow-up (75). A protective effect was also observed in the same study group (odds ratio= 0.66, CI: 0.50-0.87) after 28 years of follow-up (77). A history of tubal ligation was also associated with reduced risk among \textit{BRCA1} (odds ratio= 0.37, CI: 0.21-0.63; p=0.0003) but not \textit{BRCA2} (odds ratio= 1.00, CI: 0.38-2.66) mutation carriers in a smaller case-control study (232 matched pairs).

Interestingly, the combination of tubal ligation and OCP use appeared to provide further protection among women with a \textit{BRCA1} mutation, with an odds ratio of 0.28 (CI: 0.15-0.52) (67). In a follow-up study by the same group (McLaughlin et al discussed in Section 1.5.2), however, carriers of either \textit{BRCA1} and \textit{BRCA2} mutations showed modest reductions in risk (odds ratios of 0.80 and 0.63 respectively) but these did not reach statistical significance (66).

The reason/s for these discrepancies is unclear, although it may be relevant that in the first study a greater proportion (70%) of controls (\textit{BRCA} mutation carriers that did not go on to develop ovarian cancer) had a history of breast cancer compared to cases (35%). For instance, in a study of 449 \textit{BRCA}-associated breast cancer patients (who had not previously undergone bilateral oophorectomy), chemotherapy treatment decreased subsequent risk of ovarian cancer
(odds ratio=0.59, CI 0.29-1.21), although this association did not reach statistical significance (p=0.15) (79). This is consistent with the increased in vitro sensitivity to chemotherapeutics which cause double-strand DNA breaks (such as cisplatinum) in cells with mutations in (the DNA repair genes) \textit{BRCA1} or \textit{BRCA2} (80). Alternatively, McLaughlin et al suggested that women with breast cancer may more frequently choose to undergo tubal ligation than women without breast cancer, due to concerns of the adverse effects of pregnancy on tumour progression. Consistently, a greater proportion of women with breast cancer underwent tubal ligation in their study (21% vs. 15% of women without breast cancer) (66).

1.8. Ovarian cancer etiology: Incessant Ovulation Hypothesis

The consistent observations through epidemiologic studies that ovarian cancer risk is proportional to the lifetime number of ovulatory cycles led to the proposal by Fathalla in 1971 that ovarian cancer arises from the OSE through repeated ovulations (81, 82). This concept, commonly referred to as the ‘Incessant Ovulation Hypothesis,’ is the most enduring theory for ovarian cancer etiology, and has been generally accepted in the field until recently. Before reviewing the tenets of this theory, it is important to first outline the basics of ovulation and the ovarian cycle.

1.8.1. Brief overview of ovarian cycle

The 28 day ovarian cycle (consisting of follicular development, ovulation and luteinization) is orchestrated by complex interactions of several hormones that control the hypothalamic-pituitary-ovary axis. Briefly, pulsatile secretion of gonadotropin releasing hormone (GnRH) by neurosecretory cells within the mediobasal hypothalamus stimulates the synthesis and secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) by endocrine cells (gonadotropes) within the anterior pituitary gland. The specific gonadotropin released is partly determined by the frequency of GnRH secretion, with slow pulses favouring FSH release and rapid pulses favouring LH release. FSH and LH in turn stimulate secretion of
the steroid hormones estrogen and progesterone by steroidogenic cells within the ovary. Negative and positive feedback regulation at each step ensures that each phase of the cycle is tightly regulated.

The follicular phase of the ovarian cycle, beginning on the first day of menstruation, involves the rescue of stage 5 follicles and the eventual selection and maturation of a dominant follicle destined for ovulation. The early follicular phase is characterized by a rise in FSH, as a result of decreasing inhibition by progesterone, estrogen and inhibin at the end of the previous cycle (Figure 1.2). FSH acts to promote follicular growth and estradiol formation from androgens secreted by the thecal cells of the follicles in response to LH. The rising levels of estradiol throughout the follicular phase feeds back to inhibit further production of FSH by the anterior pituitary. Developing follicles must then compete for limiting amounts of FSH; it is believed that high expression of FSH receptors by the dominant follicle allows for its continued estradiol secretion and maturation, while the remaining follicles cease to develop and undergo atresia. Estrogen levels peak at the end of the follicular phase, exerting positive feedback on LH culminating in the preovulatory LH surge. This triggers a series of molecular events leading to follicular rupture at the ovarian surface and release of a primary oocyte into the adjacent fallopian tube (ovulation), which is the site of fertilization.

The luteal phase of the ovarian cycle begins with the (LH-dependent) formation of the corpus luteum from the components of the follicle that were not ovulated (granulosa cells, thecal cells and some surrounding connective tissue). The luteal phase is characterized by secretion of high levels of progesterone (and estrogen) by the corpus luteum under the influence of LH. Progesterone secretion peaks 5-7 days following ovulation, at which point the high levels of progesterone negatively feed back to decrease hypothalamic GnRH pulse frequency. This results in inhibition of FSH and LH secretion by the anterior pituitary and decreased expression of LH and FSH receptors by the corpus luteum. The corpus luteum undergoes atresia following lack of stimulation by LH and FSH, eventually evolving into the hormonally inactive corpus albicans (83-89).
FIGURE 1.2: Overview of the 28 day ovarian cycle. The ovarian cycle is tightly controlled by complex interactions of several hormones (hypothalamic-pituitary-ovary axis). The top two panels show the circulating levels of FSH and LH released by the anterior pituitary gland throughout the cycle, as regulated by pulsatile secretion of gonadotropin releasing hormone by the hypothalamus. The bottom two panels show the circulating levels of the ovarian steroid hormones estrogen and progesterone throughout the cycle (as regulated by FSH and LH).
1.8.2. Ovulation as an acute inflammatory response

Several lines of evidence support the idea that the process of ovulation is akin to an acute inflammatory event (76, 90-92). In short, each cycle involves infiltration by leukocytes, production of inflammatory mediators and extensive tissue remodelling leading to follicular rupture (76, 91), and nonsteroidal anti-inflammatory drugs (NSAIDs) have been shown to inhibit ovulation (93-95).

Prior to ovulation, a marked elevation of chemokines such as monocyte chemoattractant peptide 1 (MCP1, also known as chemokine (C-C motif) ligand 2) in follicular fluid leads to infiltration of periovulatory follicles by inflammatory leukocytes (96). Invading cells (mainly macrophages and neutrophils) release large amounts of inflammatory factors including the free radical nitric oxide, a mediator of interleukin-1 (IL-1)-dependent tissue remodelling and a potent vasodilator. Endothelial nitric oxide synthase (e-NOS) and inducible nitric oxide synthase (i-NOS) are also expressed by the ovarian stroma and/or thecal cells of developing follicles and are upregulated by hormonal stimulation prior to ovulation, leading to further increase in local nitric oxide levels. At the same time, the preovulatory follicle secretes large quantities of the inflammatory cytokines IL-1 and tumor necrosis factor alpha (TNFα). IL-1 enhances the expression of phospholipase A2 and cyclooxygenase 2 (COX2), enzymes responsible for production of prostaglandin E2 (PGE2). Accordingly, expression of prostaglandin endoperoxide synthase is elevated in OSE cells at the follicular apex just prior to rupture. PGE2 initiates the acute cellular events associated with inflammation, culminating in localized collagen breakdown, apoptosis at the ovarian surface and follicular rupture. Collagenolysis is first required to weaken the connective tissue layers (tunica albuginea and theca externa) of the expanding follicle to allow opening of the follicle wall. As the follicle wall thins, overlying OSE and theca externa cells at the apex of the preovulatory follicle undergo TNFα- and PGE2-dependent apoptosis. Follicular rupture then occurs through the action of collagenases and proteases characteristic of tissue responses to inflammatory reactions, including several matrix metalloproteinases. This process also requires TNFα, as
collagenolysis and ovulation are prevented following intrafollicular injection of TNFα-blocking antibodies (76, 90-92, 94, 97, 98).

Importantly, the pro-inflammatory cascade leading to ovulation also activates a compensatory anti-inflammatory cascade to limit tissue damage and promote luteinization. For instance, IL-1 and TNFα induce the expression and activity of the enzyme 11β-hydroxysteroid dehydrogenase type 1 (11βHSD type 1), which catalyzes the conversion of the glucocorticoid cortisol from its inactive precursor cortisone, in granulosa and OSE cells at the site of follicular rupture. This results in elevation of anti-inflammatory cortisol in the follicular fluid shortly before follicular rupture (91, 92, 99, 100). IL-1 also induces glucocorticoid receptor (GR) expression by OSE cells, and cortisol enhances its own formation via augmentation of cytokine-induced 11βHSD type 1 expression (99). Anti-inflammatory signalling by locally elevated cortisol is supplemented by progesterone, although the latter is mostly bound by corticosteroid-binding globulin in follicular fluid and exerts weaker anti-inflammatory effects (99).

1.8.3. Incessant Ovulation Hypothesis

As mentioned, the most enduring theory for the development of ovarian cancer has been the Incessant Ovulation Hypothesis (Figure 1.3), which states that ovarian cancer occurs through repeated cycles of ovulation-induced trauma and proliferative repair of the OSE (5, 64, 81, 101). During ovulation, the release of the oocyte into the fallopian tube requires rupture of the overlying OSE layer; this wound is then repaired by proliferation and migration of the OSE cells located adjacent to the ovulatory site. Proponents of this theory maintain that repeated bouts of apoptosis and proliferation of OSE increase the chance of genetic instability and enhance the risk for malignant transformation. Importantly, others have suggested that genetic damage to the OSE is not caused by repeated proliferation itself, but rather by exposure to the elevated levels of inflammatory mediators and reactive oxidants at the site of ovulation (76, 97, 98, 102). Consistently, potentially mutagenic 8-oxoguanine DNA adducts have been detected
FIGURE 1.3: Hypothesis that ovarian cancer arises from inclusion cysts lined by OSE.

The left panel illustrates the main tenets of the Incessant Ovulation Hypothesis, which states that ovarian cancer arises through repeated cycles of ovulation-induced trauma and proliferative repair of the OSE. Genetic damage of the OSE may occur through repeated bouts of apoptosis and proliferation, or through exposure to inflammatory mediators at the site of ovulation (not shown). OSE cells may form aggregates (aggr) and become trapped within the ovarian stroma as inclusion cysts, where direct exposure to the hormone-rich environment may contribute to Müllerian metaplasia, dysplasia and eventual malignant transformation. The right side illustrates the invagination of OSE cells into the ovarian stroma to form OSE-lined clefts as a natural process of aging. These clefts may pinch off to form OSE-lined inclusion cysts, which may also undergo dysplasia and malignant transformation similar to cysts formed through ovulatory repair. Reproduced with permission from: Ovarian surface epithelium: biology, endocrinology, and pathology. Endocrine Reviews, 22: 255-288, 2001. Copyright 2001, The Endocrine Society.
in (ovine and human) OSE cells adjacent to the site of follicular rupture, and were not observed upon prevention of ovulation in ewes. Inflammation-induced mutations could therefore conceivably be propagated by clonal expansion of OSE cells with sublethal unrepaired damage during proliferative repair of the ovulatory wound. According to the traditional hypothesis, excessive proliferation of OSE during repair of ovulatory wounds leads to formation of deep invaginations into the ovarian cortex, which can pinch off from the surface of the ovary to sit within the stroma as inclusion cysts (5, 81). In contrast to the ovarian surface where OSE cells are separated from the hormone-producing ovarian stroma by a basement membrane and a collagenous tissue layer (tunica albuginea), OSE lining inclusion cysts are directly exposed and subsequently can undergo metaplasia to become Müllerian-like (most frequently resembling tubal-like epithelium), followed by dysplasia and eventual malignant transformation (63, 69, 103). It has been proposed that differentiation to Müllerian epithelia confers a selective growth advantage via changes in hormone and growth factor receptor expression (63).

Perhaps consistent with the role of metaplasia in ovarian carcinogenesis, one study reported an increased incidence of serous differentiation within inclusion cysts of ovaries contralateral to ovarian carcinoma than in cancer-free individuals (104). In addition, gene expression profiles of the main EOC histotypes are more similar to the Müllerian epithelia that they resemble than to normal OSE cells (105). For instance, alterations observed in serous tumours (relative to normal OSE) were similarly observed in normal fallopian tube epithelium (FTE) (p=0.0042 for correlation). Similarly, alterations in endometrioid and clear cell tumours both correlated with normal endometrium (p=0.0172 and p=0.0002 respectively). Of note, samples of endocervical epithelium were not included for comparison to mucinous carcinomas, however alterations observed in this histotype correlated with normal colonic epithelium (p=0.0003) (105). A separate study in tumourigenic mouse OSE cells suggested that the distinct Müllerian differentiation patterns observed in EOC may be controlled by distinct homeobox (HOX) genes, and further that the expression of these genes could be under the influence of steroid hormones (106). Although the authors speculated that aberrant
differentiation is an early (and contributing) event in ovarian cancer development, their use of already transformed cells is problematic with respect to this claim. It is also important to note that the Müllerian (mostly tubal-like) phenotype observed in inclusion cysts is not necessarily due to altered differentiation of the OSE, and does not rule out a non-ovarian (namely fallopian tube) origin. This alternative hypothesis will be discussed in detail in Section 1.12.

There are additional problems with the theory that ovarian cancers arise within OSE-derived inclusion cysts formed through repeated ovulations. Firstly, while epidemiologic studies clearly show a protective effect for factors associated with a decreased number of ovulatory cycles (such as OCP use, parity and breastfeeding), this hypothesis cannot explain the observation that the extent of protection by OCP use and parity is greater than that expected on the basis of number of suppressed ovulations alone (63, 69). It can also not account for the enhanced risk associated with polycystic ovary syndrome (PCOS), an anovulatory condition (63, 87), and the lack of association with ages at menarche or menopause. The protective effect of tubal ligation can also not be explained, as this procedure has not been linked to reduced ovulation. In addition, the majority of inclusion cysts may simply be products of aging, rather than arising through repair of the ovulatory wound (63). Most importantly, one study reported no association between the frequency of inclusion cysts and ovarian cancer (107), and there is no conclusive evidence suggesting that these lesions are at an enhanced risk for malignant transformation.

1.9. Ovarian cancer etiology: potential protective role of progesterone signalling

The failure of the Incessant Ovulation Hypothesis to fully explain epidemiologic findings has led some investigators to propose that additional (namely hormonal) factors associated with ovulation and the ovarian cycle may contribute to ovarian cancer development (69). Most studies suggest that progesterone protects against ovarian cancer, while estrogen, androgen and gonadotropins are generally considered promoting factors. It is important to note, however,
that it is difficult to attribute a specific hormone as the major determinant of risk in these studies, as their synthetic pathways are closely linked and many associations can be interpreted as being supportive of more than one hormonal hypothesis (63).

1.9.1. The progesterone hypothesis

Multiple studies support the hypothesis that progesterone protects against ovarian cancer development (63, 69, 108). The observation that the protective effect of parity exceeds that predicted by suppression of ovulation alone led some to propose that the reduced risk may instead be attributable to the greatly (10-fold) increased concentration of maternal circulating progesterone throughout pregnancy (63, 69, 101, 108, 109). Accordingly, twin pregnancies are associated with higher levels of progesterone in comparison to singleton pregnancies (110), and multiple births (e.g. twins/triplets) may offer a greater reduction in risk among parous women, specifically for non-mucinous tumours (adjusted odds ratio=0.71, CI 0.52-0.98) (111). Interestingly, these women also have a higher incidence of double ovulation per menstrual cycle than women with a history of singleton pregnancies (111), and could therefore be predicted to be at an enhanced risk according to the Incessant Ovulation Hypothesis. The protective effect of OCPs could also be attributable to progesterone. For instance, despite the fact that up to 40% of women taking progestin-only formulations continue to ovulate, the extent of risk reduction and association with duration of use is similar to that observed for those women taking combination (estrogen+progestin) formulations associated with a greater suppression of ovulation (69). In addition, some studies have found a greater risk reduction for formulations with high compared to low progestin potency (26, 32, 108, 112). Treatment of normal OSE and ovarian cancer cells with high doses of progesterone (similar to the levels achieved during pregnancy or OCP use) induces p53-dependent cell cycle arrest and apoptosis (108, 109). These findings combined with the observed trend of decreasing risk with increasing age at first birth supports the “exfoliate” theory that exposure to high levels of
progesterone during pregnancy may rid the OSE of damaged or malignant cells (63, 101, 108, 109).

1.9.2. Progesterone receptor structure

Cells respond to progesterone through the intracellular PR. Loss of heterozygosity of the genetic locus that harbours the PR gene (11q23.3-24.3) is seen in a large proportion of ovarian cancer specimens, and is associated with a poor prognosis (101). The canonical PR is expressed as two protein isoforms, PRA and PRB, which are encoded by a single gene but arise by alternative use of two promoters (Figure 1.4). PRA and PRB proteins contain three functional domains, including the N-terminal A/B region, the highly conserved central DNA binding domain (DBD) and the C-terminal ligand binding domain (LBD) (113). The N-terminal domain of both PRA and PRB contains the ligand-independent transactivation domain AF-1, which interacts with coactivator proteins to promote activation of target genes, as well as an inhibitory domain (ID) which interacts with corepressor proteins to inhibit transcription. PRB contains an additional transactivation domain (AF-3) within the first 164 amino acids of the N-terminal portion missing from PRA, allowing interaction with a unique subset of coactivators and contributing to its stronger transactivation potential (113-117). The central DBD is composed of two zinc fingers which facilitate binding to specific progesterone response elements (PRE) in the promoter region of target genes and interaction with other transcription factors. In addition to binding to progesterone, the LBD contains an additional ligand-dependent transactivation domain (AF-2) involved in recruitment of coactivators, receptor dimerization (DIM) and interaction of inactive PR with heat shock proteins (113).
FIGURE 1.4: Structural organization of human PRA and PRB protein isoforms. The N-terminal (A/B) domain of both PRA and PRB proteins contains the transactivation domain AF-1 and inhibitory domain (ID), with PRB containing an additional activation domain (AF-3) within the first 164 amino acids. Both proteins contain the highly conserved central DNA binding domain (DBD) and the C-terminal ligand binding domain (LBD), which contains an additional transactivation domain (AF-2) and sequences involved in receptor dimerization (DIM). Numbers shown denote the amino acid position in each protein relative to PRB.
1.9.3. Progesterone receptor genomic and non-genomic signalling

In the absence of ligand, inactive PR monomers are present in the cytoplasm in association with chaperones such as heat shock protein 90 (HSP90), p23 and p59. Upon binding to progesterone, PR proteins undergo a conformational change leading to dissociation from chaperones, receptor phosphorylation and dimerization. Activated PR homodimers then translocate into the nucleus and bind to PREs in the promoter region of target genes to regulate transcription through interaction with coregulatory proteins. PR coactivators such as SRC1, steroid receptor coactivator 3 (SRC3) and cAMP response element-binding (CREB)-binding protein (CBP) facilitate transcription through interactions with general transcription machinery and promotion of local chromatin remodelling (113, 115-119).

Progesterone can also lead to rapid activation of a number of genes independent of transcriptional regulation, through interaction of cytoplasmic PR protein with components of the c-Src/MAPK, PI3K/Akt and JAK/Stat signaling pathways (114, 115, 119). For instance, cytoplasmic PR can rapidly activate c-Src following progesterone treatment of breast cancer cells, via binding of the N-terminal proline-rich motif of PR and the SH3 domain of c-Src. This subsequently leads to activation of downstream MAP kinases and cell cycle progression concomitant with cyclin D expression. This was found to be dependent on the extra-nuclear localization of PRB; while the predominantly nuclear PRA was unable to mediate progestin-dependent c-Src activation, a mutant PRA lacking the nuclear localization signal was able to do so (114). It is noteworthy that while many of the non-genomic actions of progesterone are thought to be mediated by activation of the cytoplasmic fraction of the nuclear PR (nPR), rapid progesterone responses have also been observed in cells/tissues lacking nPR such as T lymphocytes, platelets and PR knockout mice (119), leading to the discovery of the (as yet uncharacterized) membrane-associated PR (116).
1.9.4. Progesterone receptor isoforms

Finally, it is important to note that PRA exhibits differential functional properties compared to the full-length PRB isoform, including regulation of distinct genes and differential effects on target tissues in knockout mice (113, 117, 120). In addition, PRA has been shown to act as a model-, cell- and promoter-specific hormone-dependent repressor of PRB-mediated transcription in cells where PRA itself had minimal transactivation activity (121-123). While PRA and PRB are co-expressed at roughly equivalent levels in most target tissues (117), the relative expression of these two isoforms has been shown to vary in reproductive tissues as a result of development, hormonal status and malignant transformation (113). While the expression of both isoforms is upregulated by estrogen, PRB has been found to be preferentially induced in ovarian and breast cancer cell lines (124, 125). However, estrogen increases PRA to a greater extent than PRB in the chicken oviduct, suggesting that regulation of PR isoforms by estrogen is likely cell, tissue and species-specific (124). Particularly relevant to this thesis, while PRB (protein and mRNA) was similarly expressed in normal OSE and ovarian cancer in a study by Akahira et al, PRA was significantly downregulated in malignant tissues, resulting in a lower expression of PRA compared to PRB in all EOC histotypes (126).

1.10. Ovarian cancer etiology: potential role of androgens, estrogens and gonadotropins

1.10.1. The androgen hypothesis

In contrast to progesterone, it has been proposed that ovarian cancer risk is increased by excessive stimulation of OSE by androgens, estrogens and/or gonadotropins (63, 69, 101, 108). In the premenopausal ovary, LH-stimulated thecal cells surrounding growing follicles secrete large quantities of the androgens androstenedione, testosterone and dehydroepiandrosterone (63, 85). Although the follicular fluid of the dominant (ovulating) follicle is largely estrogenic following FSH stimulation of granulosa cells prior to ovulation, smaller
follicles and post-menopausal ovarian stromal cells continue to secrete androgens, suggesting that OSE cells (particularly within inclusion cysts) would be appreciably exposed (69).

Potentially consistent with the “androgen hypothesis,” OCPs suppress ovarian androgen production and elevated levels of circulating androgen are observed in women with PCOS, a condition which has previously been associated with increased risk (63, 69, 87, 108, 109). It is important to note, however, that a recent large population-based case-control study did not find evidence that PCOS increases the overall or histotype-specific risk of ovarian cancer, with the exception of serous borderline (LMP) tumours (127). A significantly increased risk has also been observed in women with endometriosis that had been treated with a synthetic androgen compared to those taking a GnRH agonist that inhibits ovarian androgen production, although the histology of included cases were not indicated (128). In addition, androgen increases proliferation of normal OSE and ovarian cancer cells in vitro, likely by interfering with transforming growth factor-beta (TGFβ)-mediated growth inhibition (63, 109, 129). Finally, women who inherit an AR allelic variant with fewer polyglutamine (CAG) repeats develop (both sporadic and BRCA-associated) ovarian cancer an average of 7.2 years earlier (CI 2.3-12.1 years, p=0.0004) than those women with a long CAG repeat length (130). A short AR allelotype (<19 CAG repeats) was also associated with shorter progression-free (5.5 vs. 19.4 months, p<0.0001) and overall (9 vs. 32.6 months, p=0.0007) survival in a study of 77 patients with EOC (131), although the association by histological subtype was not indicated. Shorter alleles lead to a more stable AR protein with increased p160 coactivator binding and enhanced transcriptional activity, suggesting that AR signalling may promote and/or accelerate tumour progression (32, 37). It is presently unclear how these observations can be reconciled with the stimulation of AR transactivation activity by the tumour suppressor proteins BRCA1 and BRCA2 described in Section 1.4.4. One possibility is that the impact of BRCA1 on AR signalling may be influenced by duration of androgen exposure, as chronic androgen treatment revealed an increased expression of AR-dependent genes in primary OSE cells derived from BRCA1/2 mutation carriers compared to control patients (132).
1.10.2. The estrogen hypothesis

It has been proposed that excessive stimulation of OSE by estrogens contributes to ovarian carcinogenesis (63, 69, 101). In the premenopausal ovary, FSH-stimulated granulosa cells secrete large quantities of the estrogens 17β-estradiol and estrone by conversion of thecal cell-derived androgen (69, 85). This leads to a 100-fold higher ovarian tissue level of estrogens compared to the circulation, with an even higher concentration found in the follicular fluid of ovulatory follicles which bathes the OSE during ovulation (63, 101). In general, in vivo proliferation of OSE generally occurs at times in the ovarian cycle when estrogen levels are high. The increased mitotic activity likely enhances the risk of mutations, which could propagate with additional proliferation in future cycles. Therefore, it is proposed that the high levels of estrogen present in follicular fluid and the ovarian stroma lead to genetic instability of OSE cells at the site of follicular rupture and within inclusion cysts, promoting malignant transformation (63, 69, 101).

Potentially consistent with the estrogen hypothesis, decreased endogenous ovarian estrogen production occurs with OCP use, an established protective factor for ovarian cancer in premenopausal women (63, 69). In addition, endometriosis, an established risk factor for endometrioid and clear cell ovarian carcinoma, is associated with increased local production of estradiol (63). Estrone and 17β-estradiol have been shown to increase proliferation of OSE and ovarian cancer cells in vitro, and estrogen receptors (ERα and ERβ) are expressed by both normal and malignant cells (63, 101, 109, 133). Mounting evidence also suggests that oxidative metabolites of estrogen are genotoxic and may directly damage DNA (108). Potentially inconsistent with the estrogen hypothesis, pregnancy, despite being protective for ovarian cancer, is associated with a greatly (100-fold) increased concentration of maternal circulating estrogen (63, 69). Finally, epidemiologic studies of estrogen-based HRT largely suggest that estrogens act to promote the growth of pre-existing tumours rather than contribute to malignant transformation directly (63, 73). This is illustrated by the findings of the UK Million Women
Study (Section 1.6), in that current but not past users of HRT containing unopposed estrogen were at an enhanced risk for ovarian cancer (73).

1.10.3. The gonadotropin hypothesis

The "gonadotropin hypothesis," as stated by Cramer and Welch in 1983, postulates that ovarian cancer develops through excessive stimulation of OSE cells by the pituitary gonadotropins LH and FSH (63, 108, 134). This is thought to occur either through direct activation of gonadotropin-responsive genes that contribute to malignant transformation, or indirectly through stimulation of ovarian steroidogenesis. Potentially consistent with this hypothesis, pregnancy and OCPs both suppress pituitary secretion of gonadotropins, with lower basal and peak stimulation of the OSE (63, 69, 108). In addition, circulating levels of LH are elevated in women with PCOS (63, 87, 108), and the incidence of (sporadic) ovarian cancer peaks 10-20 years following menopause when levels of gonadotropins are at their highest (109). Accordingly, GnRH has been shown to exert anti-tumour effects, likely through desensitization/downregulation of pituitary GnRH receptors and subsequent reductions in gonadotropin secretion and ovarian steroidogenesis (108). Potentially inconsistent with this hypothesis, women with ovarian cancer have not been shown to have elevated blood levels of FSH or LH compared with controls (63). Also, age at menopause has not been shown to substantially affect risk, despite the fact that women with premature ovarian failure or early menopause have elevated gonadotropin levels (76). In addition, while some studies have observed a proliferative effect of FSH and LH on human OSE and ovarian cancer cells, this has not been consistently observed (63, 108, 109). Finally, ovarian tumours have been observed in (anovulatory) FSH receptor knockout (FORKO) mice (135-137), suggesting that neither incessant ovulation nor FSH receptor signalling are required for ovarian carcinogenesis. It is important to note, however, that the ovarian androgens were significantly elevated in mutant mice and the majority of lesions appear to be sex-cord stromal tumours or benign epithelial cysts, therefore the direct impact of FSH signalling on ovarian carcinomas is unclear.
1.11. Ovarian cancer etiology: The Inflammation Hypothesis

1.11.1. Tubal ligation and the Inflammation Hypothesis

As mentioned in section 1.7, a history of tubal ligation is associated with a decreased risk of ovarian cancer; this is despite uninterrupted ovulation and a lack of significant changes in ovarian hormone levels. For instance, a retrospective study found that women who had previously undergone a tubal ligation procedure showed no difference in either the duration of the follicular and luteal phases, or mid-luteal levels of progesterone. However, lower preovulatory and mid-luteal levels of LH and 17β-estradiol were reported (138). Importantly, this was not observed in a recent prospective study consisting of 118 fertile women undergoing tubal ligation (either by the bipolar cautery or Hulka clip method) and 57 fertile controls. Over a period of two years, no significant changes were found in luteal phase levels of serum or urinary estrogen or progesterone in sterilized women compared to their baseline levels or age-matched unsterilized controls (139). Some authors have stated that the protective effect of tubal ligation supports the hypothesis that ovarian cancer originates in the fallopian tube (not the ovary), and that tubal ligation interrupts the transport of these cells to the ovarian surface (140). This hypothesis will be explored in detail in Section 1.12. An alternative explanation for the protective effect of tubal ligation that will be discussed here is that it reduces the potential for ovarian inflammation (76).

The relevance of inflammation in ovarian cancer is highlighted by the decreased risk associated with the use of NSAIDs. Importantly, a recent study reported a protective effect of NSAIDs in never users (odds ratio= 0.58, CI: 0.42-0.80), but not in ever users (odds ratio= 0.98, CI: 0.71-1.35), of OCPs (p for interaction=0.03). Similarly, NSAIDs reduced risk among nulliparous (odds ratio= 0.47, CI: 0.27-0.82) but not parous (odds ratio= 0.81, CI: 0.64-1.04) women (p for interaction=0.05), suggesting that NSAIDs are particularly beneficial to women in higher risk groups (141). Accordingly, additional risk factors of ovarian cancer not discussed in the previous sections, including talc exposure, endometriosis and pelvic inflammatory disease.
(PID), are not directly linked to ovulation or reproductive hormones, and are instead associated with local pelvic inflammation. Tubal ligation may thus exert protection against ovarian cancer by blocking the transport of genital tract irritants from the lower genital tract to the ovary, thereby limiting the exposure of OSE cells to initiators of inflammation (76). The majority of epidemiologic studies have reported that talc use (specifically exposure described as a ‘dusting of the perineum’) is associated with an increased risk of ovarian cancer. Interestingly, tubal ligation was found to protect against the effect of talc in one study; while talc use increased risk by 30%, a 50% risk reduction was observed for those women who also had a history of tubal ligation (76).

Similarly, endometriosis (the ectopic implantation of endometrial tissue) leads to a local inflammatory reaction and is associated with an increased risk of (histotype-specific) ovarian cancer. One study reported the presence of endometriosis in 40% of women diagnosed with stage I endometrioid or clear cell carcinoma (76). Perhaps consistent with the protective role of tubal ligation in endometriosis-associated ovarian carcinogenesis, a case-control study of risk factors subdivided by histological type reported the greatest protective effect of tubal ligation for invasive endometrioid carcinomas (odds ratio= 0.2, CI: 0.1-0.6) compared to all histotypes combined (odds ratio= 0.7, CI: 0.5-1.0) (142). Finally, PID involves the inflammation of the endometrium, fallopian tubes and ovaries, and results from the transport of sexually transmitted infections from the lower to upper genital tract (76). A large case-control study in Toronto (450 cases, 565 controls) reported that women with a previous episode of PID had an increased risk of ovarian cancer (odds ratio= 1.5, CI: 1.1-2.1), and a trend for increasing risk with increasing numbers of PID episodes was observed (143). Notably, several studies suggest that tubal ligation may protect against PID (144); it would therefore be interesting to directly assess the impact of tubal ligation on the risk associated with PID.
1.11.2. Inflammatory signalling is primarily mediated by NFkB

Pro-inflammatory signalling is primarily mediated by the ubiquitous transcription factor nuclear factor-kB (NFkB) (145). The NFkB protein family consists of five members (RelA, RelB, c-Rel, NFkB1/p50 and NFkB2/p52), which form various hetero- and homodimers according to cell type and the nature and duration of the inducing stimulus (146, 147). The most common NFkB dimer consists of p50 and the transcriptionally active v-rel reticuloendotheliosis viral oncogene homolog A (RelA) (145, 148, 149). RelA is characterized by an N-terminal Rel homology domain (required for DNA binding, dimerization and nuclear localization) and a C-terminal transactivation domain (149, 150).

In the absence of ligand, monomers of NFkB subunits reside in the cytoplasm in an inactive state through association with IκBα inhibitory proteins (Figure 1.5). Activation of signalling is rapidly triggered by the binding of pro-inflammatory cytokines, most notably TNFα, to specific cell surface receptors. Ligand stimulation activates a kinase cascade ultimately resulting in the recruitment and activation of the canonical IκB-kinase (IKK) complex, leading to the phosphorylation, ubiquitination and proteolytic degradation of IκBα. This releases the bound RelA and p50 subunits, whereupon they translocate to the nucleus, dimerize and bind to specific NFkB response elements in the promoter regions of target genes (145, 147, 149, 151, 152). Transcription is then promoted by recruitment of coactivator proteins by RelA, including the histone acetyltransferase CBP (152, 153). The transactivation potential of RelA is enhanced by phosphorylation (of serine residue 276), in both the cytoplasm by protein kinase A (PKA) following release from IκBα, and in the nucleus, by mitogen- and stress-activated protein kinase-1 (MSK-1) (152).

NFkB induces several pro-inflammatory genes, including cytokines (e.g. IL-1, TNFα, IL-6) and their respective receptors, chemokines (e.g. IL-8, MCP1) responsible for recruitment of inflammatory leukocytes, cell adhesion molecules (e.g. ICAM1, VCAM1), matrix metalloproteinases and the inflammatory enzymes COX2 and iNOS (100, 147, 151). Production of pro-inflammatory cytokines by NFkB generates a positive feedback loop, leading
FIGURE 1.5: Schematic representation of canonical NFκB pathway. In the absence of ligand, monomers of NFκB subunits (RelA and p50) reside in the cytoplasm in an inactive state through association with IκBα inhibitory proteins. Binding of pro-inflammatory cytokines to their specific cell surface receptors activates the canonical IκB-kinase complex, leading to the phosphorylation, ubiquitination and proteolytic degradation of IκBα. This releases the bound RelA and p50 subunits, whereupon they translocate to the nucleus, dimerize and bind to specific NFκB response elements to activate transcription (through RelA-dependent recruitment of coactivator proteins such as CBP). RelA transactivation activity is enhanced by cytoplasmic phosphorylation by PKA (1) and nuclear phosphorylation by MSK-1 (2).
to amplification of the inflammatory response (145). NFκB is constitutively activated in many human cancers, and has been shown to be the critical link between chronic inflammation and tumour development in some model systems (147, 151). Chronic activation of the inflammatory response by NFκB may increase tumour risk by induction of genomic instability, through production of potentially mutagenic reactive oxygen species. Furthermore, NFκB-dependent induction of genes involved in cell cycle progression (e.g. cyclin D1 and c-Myc) and prevention of apoptosis (e.g. Bcl-XL and X-IAP), would promote the survival of damaged cells (76, 95, 102, 147, 151, 154, 155).

1.11.3. Anti-inflammatory signalling is primarily mediated by the glucocorticoid receptor

While cytokines such as TNFα and IL-1 activate pro-inflammatory signalling by NFκB dimers, they also activate compensatory anti-inflammatory signalling by promoting local formation (Section 1.8.2) and systemic secretion of glucocorticoids. Release of inflammatory cytokines directly stimulates the hypothalamic-pituitary-adrenal (HPA) axis (156), which controls the concentration of circulating glucocorticoids. Briefly, hypothalamic secretion of corticotropin-releasing hormone (CRH) stimulates the secretion of adrenocorticotropic hormone (ACTH) from the anterior pituitary corticotrope cells, which in turn stimulates synthesis and secretion of cortisol by the adrenal cortex. Glucocorticoids exert a negative feedback effect on this pathway through decreased secretion of both CRH and ACTH (149, 157). Glucocorticoids have been shown to inhibit many aspects of inflammation, including the migration and function of leukocytes and the production and downstream signalling of pro-inflammatory cytokines. Glucocorticoids have also been shown to induce cell-type specific apoptosis of monocytes, macrophages and T lymphocytes (100, 145, 148, 149). Unlike other steroid hormones discussed in previous sections, the role of glucocorticoids in ovarian cancer has not yet been investigated.

Cells respond to glucocorticoids through the intracellular GR, a member of the steroid and thyroid receptor superfamily. Two major isoforms (GRα and GRβ) of the GR exist that
result from differential splicing of the same gene located on chromosome 5 (5q31) (158). The
classic GRα isoform contains three functional domains, including an N-terminal transactivation
domain (AF-1) required for association with basal transcription factors and transcriptional
enhancement, a central DBD, and a C-terminal LBD (145, 158). The DBD contains two
conserved zinc finger regions involved in GR dimerization, binding to recognition sites on the
chromatin (glucocorticoid response elements, GREs), and activation and repression of
transcription, while the LBD is involved in binding to hormones (cortisol), chaperone proteins
and transcriptional coactivators (145).

In the absence of ligand, GRα monomers reside in the cytoplasm in an inactive state
through association with chaperones including HSP90, which act to maintain GR in a
conformation favourable for high-affinity ligand binding (Figure 1.6) (145, 149, 158). Upon
binding cortisol (which enters the cell by free diffusion through the cell membrane), activated
GRα undergoes a conformational change that leads to dissociation from co-chaperone
proteins, homodimerization and translocation into the nucleus. Interaction of GRα dimers with
GREs in the promoter region of target genes results in stimulation of gene transcription by
recruitment of coactivator proteins including CBP, GRIP1 and SRC1 (145, 149, 157-160).
Through this mechanism (termed ‘transactivation’), GRα induces several anti-inflammatory
genes, including but not limited to dual specificity phosphatase 1 (DUSP1), glucocorticoid
inducible leucine zipper (GILZ), annexin A1, IL-1 receptor antagonist and the anti-inflammatory
cytokines IL-10 and TGFβ (145, 149, 159).

The primary mechanism by which GRα inhibits inflammatory signalling, however, is
through repression of RelA activity independent of DNA binding (‘transrepression’), resulting in
suppressed synthesis of inflammatory mediators (145, 148, 150). It is important to note that
GRα and RelA are mutually inhibitory, as RelA can similarly decrease GRα-mediated
transcription (145, 148). Several mechanisms have been proposed for cross-repression of GRα
and NFκB, including direct interaction of (ligand-activated) GRα and RelA monomers (145, 148,
149). This interaction may occur in the cytoplasm, where the catalytic subunit of PKA (PKAc)
**FIGURE 1.6: Schematic representation of anti-inflammatory signalling by GR.** In the absence of ligand, GRα monomers reside in the cytoplasm in an inactive state through association with chaperone proteins such as HSP90. Upon activation by glucocorticoids, GRα dissociates from HSP90 and translocates to the nucleus where GRα homodimers interact with specific glucocorticoid response elements to activate transcription of anti-inflammatory genes through recruitment of coactivator proteins such as CBP (1). GR also inhibits RelA activity, leading to decreased transcription of pro-inflammatory genes. Mechanisms of transrepression may include interaction of ligand-activated GRα and RelA monomers in the cytoplasm, where PKA mediates their mutual inhibition (2). Alternatively, nuclear GRα homodimers may interact with RelA at NFκB response elements (3), where GR can interfere with phosphorylation of RNA polymerase II. GR may also limit the transactivation potential of RelA through competition for coactivator proteins or through nuclear export of the essential RelA kinase MSK-1 (not shown).
mediates their mutual inhibition (150). Alternatively, nuclear interaction of GRα and RelA at κB response elements in the promoters of target genes can prevent RelA-dependent transcription by interference of RNA polymerase II phosphorylation (145, 161, 162). GRα and RelA may also compete for limiting amounts of coactivators (most notably CBP), which are required for optimal transactivation potential of both proteins (145, 163, 164). In addition, activated GRα can counteract the recruitment of the essential RelA kinase MSK-1 to inflammatory gene promoters, by triggering export of activated MSK-1 from the nucleus to the cytoplasm (162). Altogether, GRα appears to inhibit RelA-dependent transcription of inflammatory genes through several (likely context-dependent) mechanisms, ultimately leading to the resolution of the inflammatory process.

Unlike GRα, GRβ is constitutively localized in the nucleus, does not bind glucocorticoids and is transcriptionally inactive (145, 158, 160). In cell culture, overexpressed GRβ acts as a dominant negative inhibitor of GRα transactivation activity. In fact, GRβ can bind GRE-containing DNA independent of ligand stimulation and with greater affinity than GRα. GRβ has also been shown to inhibit GRα-mediated repression of NFκB and activator protein-1 (AP-1), an additional regulator of inflammatory signalling (160). Interestingly, GRβ expression is selectively induced by the pro-inflammatory cytokines TNFα and IL-1 in inflammatory cell types via an NFκB binding site in the GR promoter, resulting in resistance to glucocorticoids (145, 160).

1.12. Ovarian cancer etiology: fallopian tube as a source of high-grade serous carcinoma

Despite the many theories of ovarian cancer development outlined in the previous sections, the cell of origin of ovarian cancer remains unresolved. In 1999, Louis Dubeau proposed that ovarian carcinomas may not arise from the OSE, but rather from components of the secondary Müllerian system (e.g. paraovarian/paratubal cysts, rete ovarii, endosalpingiosis,
endometriosis and endomucinosis) (165). Other groups have postulated alternative cells of origin. Most germane to this thesis is the proposal that ovarian SerCa is derived from the epithelium of the distal fallopian tube (17, 103, 140). Evidence leading to the formulation of this novel hypothesis will be discussed in the remaining sections.

1.12.1. Anatomy and physiology of the normal fallopian tube

The human fallopian tubes are paired organs derived from the paramesonephric duct (Müllerian duct) that bridges the uterus and ovaries, with crucial roles in ovum pick-up, ovum transport, facilitation of fertilization and support of pre-implantation embryo development (166). Fallopian tubes consist of three layers: the (innermost) mucosal, comprised of simple columnar ciliated and secretory epithelial cells, the muscularis externa and the (outermost) serosa. Each tube can be divided into four main segments: the interstitial portion (within the uterus), the isthmus, the ampulla and the infundibulum (Figure 1.7). The infundibulum terminates in freely mobile finger-like projections, termed fimbriae, which are covered in FTE cells and directly contact the adjacent ovarian surface and peritoneal cavity. The ciliated epithelium of the fimbriae cover the growing follicle during ovulation, and transport of the ruptured ovum occurs through gentle sweeping movements of the fimbriae and the ciliary action of the FTE (167).

Secretory FTE cells lining the tube support oocyte survival and fertilization by synthesis and secretion of various factors including oviducal glycoprotein (OGP) (168).

Ciliated and secretory FTE cells express both ER and PR and undergo cyclic changes in growth and differentiation throughout the ovarian cycle; these changes are most evident in the fimbriae and least apparent in the isthmus (166, 169). During the follicular phase (see Section 1.8.1), estrogen promotes formation of new cilia (10% of cells) and increases the height and mitotic activity of FTE cells (169, 170). Accordingly, treatment of primary FTE cultures with 17β-estradiol induces differentiation of one-third of cells from an immature secretory-like to a ciliated phenotype. The FTE becomes deciliated following the decline and loss of ovarian estrogen production at menopause, unless estrogen replacement therapy is
FIGURE 1.7: Segments of the human fallopian tube. Shown is a single fallopian tube, uterus and ovary with the segments of the fallopian tube labelled.
provided (166). Estrogen has also been found to increase proliferation of primary FTE cells (obtained from the ampullary portion of the fallopian tube during the follicular phase) in an epidermal growth factor (EGF)-dependent manner (171). Maximal secretory activity (as measured by presence of OGP mRNA) is also observed at late follicular phase when estrogen levels are high; OGP secretion is dependent on estradiol and is suppressed by co-treatment with progesterone (166, 168). During the post-ovulatory luteal phase, progesterone leads to deciliation, loss of mitotic activity and a decrease in cell height (169, 170, 172). Further deciliation and atrophy of FTE is observed during pregnancy (169).

1.12.2. Similarity of fallopian tube and ovarian serous carcinoma

Fallopian tube carcinoma is a rarely diagnosed gynaecologic malignancy (0.3% of cases), which typically presents at an advanced stage with an associated 5-year overall survival rate of 30% (173, 174). Carcinomas are thought to arise from the secretory epithelial cells (175), and tumours can implant throughout the peritoneal cavity by transcelomic exfoliation (176). Similar to ovarian carcinoma, serous carcinomas are the most common histological type observed (up to 90% of cases), with the majority of these cases being of high histologic grade (174, 176, 177). A recent multi-institutional matched case-control study of stage III/IV serous carcinomas diagnosed as being of tubal (n=46) or ovarian (n=92) origin reported identical overall (59%) and very similar progression-free (26% vs. 21%) survival after 3 years of follow-up (178), suggesting that tubal and ovarian SerCa show similar clinical response to treatment. Importantly, >90% of patients in each group received standard combination (taxane + platinum) chemotherapy following cytoreductive surgery, and each tubal carcinoma patient was matched with two ovarian cancer patients according to age, year of diagnosis, site of primary surgery, residual disease, and grade of disease. Also similar to ovarian SerCa, tubal carcinomas show an extremely unstable phenotype and frequent (81%) alterations in p53 (176, 177). Importantly, comparative genomic hybridization on 20 primary fallopian tube carcinomas detected frequent gains at 3q (70%) and 8q (75%), regions
previously shown to be similarly altered in ovarian SerCa. Also, losses were frequently observed at 4q, 5q, 8p and 18q, two of which (8p and 18q) have previously been observed in ovarian SerCa. The similar frequency and pattern of chromosomal alterations detected in tubal and ovarian SerCa supports the notion that these tumours (at least) share a common molecular pathogenesis (179).

Risk factors identified for ovarian cancer also extend to fallopian tube cancers. Of particular note, it has recently been confirmed that greater than 15% of tubal cancers are associated with underlying germline mutations in BRCA1 or BRCA2 (27, 173, 174). Importantly, detailed study of two BRCA1-associated tumours demonstrated loss of the wild type BRCA1 allele in both cases, directly implicating BRCA1 in tubal carcinogenesis (173). Accordingly, inheritance of a BRCA1 mutation was associated with a 120-fold increased risk for primary fallopian tube cancer in a large retrospective analysis (27). Similar to hereditary ovarian carcinoma, BRCA1/2-associated tubal carcinomas are predominantly high-grade serous tumours, with an early age at onset and frequent p53 abnormalities (>70%). Both BRCA-associated and sporadic tubal cancers are predominately located in the distal end of the fallopian tube adjacent to the ovarian surface (27, 180).

In addition to BRCA mutations, tubal carcinomas have been associated with chronic inflammation (177), a factor that seems to be relevant for ovarian carcinoma (Section 1.11). Reproductive factors such as OCP use, parity and breastfeeding are also associated with decreased risk of primary fallopian tube cancer (176, 181, 182). The inverse association between fallopian tube cancer risk and anovulatory factors (181) argues against the traditional Incessant Ovulation Hypothesis that physical damage to the OSE during ovulation underlies ovarian SerCa development, as this hypothesis cannot account for similar tumours in the fallopian tube. Collectively, both hereditary and sporadic tubal carcinomas are strikingly similar to those tumours observed in the ovary.
1.12.3. Lack of ovarian pre-malignant lesions in BRCA1/2 mutation carriers

The majority of women who test positive for the presence of a BRCA1 or BRCA2 mutation elect to undergo prophylactic bilateral salpingo-oophorectomy (BSO) after completion of childbearing to reduce their future cancer risk. According to a retrospective study of 493 BRCA mutation carries (245 of which underwent surgery), prophylactic BSO reduces the risk of ovarian, fallopian tube or primary peritoneal cancer by 98% (35). Given that mutation carriers are genetically predisposed to SerCa development, a proportion of these prophylactic specimens are expected to harbour pre-malignant histological or molecular changes involved in serous carcinogenesis.

Despite the enduring belief that ovarian SerCa arises from the OSE, intensive study of prophylactically removed ovaries has thus far failed to reveal reproducible pre-malignant epithelial changes (17, 183-187). Although early, non-blinded studies reported an increased proportion of potentially preneoplastic histological features (including but not limited to surface papillomatosis, surface epithelial stratification, deep cortical invaginations and epithelial inclusion cysts) in the ovaries of high-risk women (188), these results have been challenged in follow-up studies blinded to BRCA mutation status (183, 184, 189, 190). For instance, Piek et al reported a similar incidence of ovarian inclusion cysts in mutation carriers (31/87, 36%) and controls (11/40, 27%) (190). In addition, despite frequent alteration of p53 in high-grade ovarian SerCa, histologically normal ovaries from BRCA mutation carriers do not show evidence of altered p53 status (as indicated by accumulation of p53 by IHC) (183, 187). Primary cultures derived from OSE of mutation carriers also fail to exhibit enhanced growth potential or altered morphology compared to those obtained from control patients (186).

1.12.4. Fallopian tube lesions are commonly found in BRCA1/2 mutation carriers

In stark contrast, putative precursor lesions and clinically occult carcinomas have frequently been identified in prophylactically removed fallopian tubes (17, 174, 191-194). The majority of these lesions are found in the fimbriated end of the fallopian tube adjacent to the
ovary, frequently in the absence of ovarian pathology. The most surprising observation has been the high incidence (4-17%) of occult carcinomas involving the fallopian tube, despite the apparent rarity of primary fallopian tube carcinomas (27, 192-197). In the largest study to date, Finch et al reported the presence of 7 early occult cancers in 159 (4.4%) of BRCA1/2 mutation carriers undergoing prophylactic BSO at the University Health Network in Toronto. Six of these cancers involved the fallopian tube (including 5 located in the fimbria), and 3 involved the fallopian tube but not the ovaries. In addition, the incidence of occult cancer was higher in BRCA1 (6/94, 6.4%) compared to BRCA2 (1/65, 1.5%) mutation carriers (193), possibly reflecting the decreased penetrance of BRCA2 mutations and the fact that the average BRCA2-associated cancer occurs a decade later than the average age at surgery (48.8 years) in this study. A separate study by Crum and coworkers analyzed in toto the ovaries and fallopian tubes from 13 consecutive prophylactic BSO procedures (average age 55 years) following an index case of fimbrial SerCa. Four early tubal SerCas were detected in the fimbria or ampullary region (in a segment just proximal to the fimbria), including three non-invasive (intraepithelial) lesions (hereafter referred to as tubal intraepithelial carcinoma, TIC). Notably, all tubal SerCas showed co-localized positivity for p53 and the proliferation marker Ki-67, and no ovarian carcinomas were detected (194). Recently, Shaw et al confirmed these findings in a large independent case set, with BRCA mutation carriers showing an increased frequency of TIC (15/176, 8%) compared to controls (2/64, 3%) in a blinded review (197). The relevance of TICs in serous carcinogenesis was further illustrated by a follow-up study by the Crum group, which examined the fallopian tubes of 55 consecutive pelvic SerCa cases unselected for family history or BRCA mutation. Importantly, 30/42 (71%) of cases classified as primary ovarian SerCa showed endosalpinx (inner tubal lining) involvement, while 20/42 (48%) contained co-existing (primarily fimbrial) TIC lesions. TIC was also detected in 5/8 (63%) of cases diagnosed as primary peritoneal SerCa, implicating the FTE as a plausible source for these tumours as well. Importantly, analysis of five “ovarian” SerCas and co-existing TIC lesions revealed
identical mutations of TP53, clearly suggesting that these two lesions are causally related (196).

In addition to early tubal (invasive and non-invasive) cancers, a large proportion (up to 50%) of prophylactically removed fallopian tubes contain pre-malignant epithelial changes that may precede SerCa development (180, 190, 191, 197, 198). Most notably, the Crum group have described foci of strong p53 immunopositivity ("p53 signatures") in benign appearing FTE. These genetic lesions share many features with TICs, including fimbrial location, frequent TP53 mutations and immunopositivity, secretory cell phenotype (as indicated by HMFG2 positivity) and evidence of DNA damage (as indicated by co-localization of γ-H2AX). Unlike TICs, p53 signatures do not exhibit marked nuclear atypia or proliferative activity (180). Although p53 signatures were equally common in non-malignant FTE from BRCA mutation carriers and controls in this and two additional studies (197, 198), they were more frequently observed in fallopian tubes also containing TICs (180). In addition, a shared TP53 mutation was detected in one case with a contiguous p53 signature and TIC lesion. Importantly, Folkins et al reported the presence of p53 signatures in 29/75 (38%) of BRCA1/2-mutated fallopian tubes, while no lesions were detected in ovarian cortical inclusion cysts in the same patients (198). Collectively, these observations strongly suggest that the cell of origin for a significant proportion of both hereditary and sporadic 'ovarian' SerCa is the FTE, not OSE.

1.12.5. An alternative model of “fimbrial-ovarian” serous neoplasia

Given the similarities of tubal and ovarian SerCa and the frequency of occult carcinomas and putative precursors discovered in the fallopian tubes of BRCA mutation carriers, an alternative model of SerCa has been proposed in which the FTE plays a major role (Figure 1.8). According to the model of “fimbrial-ovarian” serous neoplasia recently articulated by Crum et al (17), the first step of this pathway entails genotoxic injury to the secretory epithelial cells of the distal fallopian tube, which may lead to unrepaired DNA damage, cell cycle arrest and TP53 mutations in a subset of cells. Clonal expansion of a TP53-mutated cell
Normal FTE
- Genotoxic damage
- Cell cycle arrest
- p53 mutation

p53 signature
- Additional genetic damage
- Re-initiation of proliferation
- Malignant transformation

TIC
- Tumour expansion
- Local invasion
- Exfoliation onto ovarian surface

SerCa
FIGURE 1.8: FTE as the cell of origin of ‘ovarian’ SerCa. The first step in this pathway (as articulated by Crum et al) entails genotoxic injury to the secretory epithelial cells of the distal fallopian tube (‘ep’), leading to unrepaired DNA damage, cell cycle arrest and TP53 mutations in a subset of cells (p53 signature, indicated by p53 immunopositivity in 2nd panel inset). Re-initiation of cell proliferation in a subset of p53 signatures (through potential BRCA1/2-dependent mechanisms) may lead to the development of an early non-invasive SerCa (tubal intraepithelial carcinoma, TIC). Aggressive subclones of TICs may locally invade the underlying tubal stroma to present as primary fallopian tube SerCa or exfoliate onto the closely associated ovarian surface to present as ‘ovarian’ SerCa. Stromal cells are indicated by ‘st’ in the top three panels. Modified from: The distal fallopian tube: a new model for pelvic serous carcinogenesis. Curr Opin Obstet Gynecol, 19: 3-9, 2007.
would then result in focal accumulation of p53 detectable by IHC (p53 signature). The similarly frequent occurrence of p53 signatures in the distal FTE of BRCA1/2 mutation carriers and control patients (who are at a much lower risk for ovarian cancer), suggests that inheritance of a BRCA1/2 mutation may increase the risk of malignant transformation rather than formation of the precursor itself (17, 180). This is consistent with precursor lesions in other systems (such as the colonic epithelium), in that they are relatively common and do not invariably progress to malignancy (17). The second step of this pathway involves re-initiation of cell proliferation (by an as yet unidentified mechanism likely related to the presence of a BRCA mutation) in a subset of these p53 signatures, leading to the development of a TIC. In the final step, highly aggressive subclones of TICs may locally expand and invade the underlying tubal stroma, presenting as primary fallopian tube carcinoma. Alternatively, a TIC lesion may exfoliate onto the closely associated ovarian surface (or peritoneal cavity) and present as primary ovarian (or peritoneal) carcinoma (17, 186).

A few explanations for the fimbrial location of the majority of tubal lesions have been suggested. Firstly, the abundance of surface area in this region may mathematically increase the risk that a neoplasm will arise. Secondly, there may be (as yet undiscovered) differences in the mucosa of the fimbria that make them inherently more susceptible to carcinogenesis compared to the proximal segments of the fallopian tube. Thirdly, the proximity of the fimbria to the peritoneal cavity and ovarian surface may be involved (17, 180, 194). For instance, the association of ovulatory factors with fallopian tube (and ovarian) carcinoma risk suggest that the intimate association of the fimbrial FTE with the ovulatory follicle may contribute to genotoxic injury, as these epithelial cells would be exposed to the hormone- and prostaglandin-rich follicular fluid at the time of ovulation (180, 181).

Another question raised by prophylactic BSO findings is how to reconcile the high frequency of early tubal vs. ovarian lesions with the rare diagnosis of primary fallopian tube carcinoma. The most likely answer is that the true incidence of tubal carcinoma is grossly underestimated, as it can be difficult to determine tissue of origin in its advanced stages and
can consequently be misdiagnosed as primary ovarian cancer (173, 176). In fact, to apply the diagnosis of a primary tubal carcinoma, very strict histological criteria must be met. For instance, the main tumour burden must be in the tube (with ovaries either appearing normal or containing less tumour), there must be mucosal involvement, and a transition from benign epithelium to carcinoma should be observed (174, 176, 177). Although it is not possible to definitively prove the tubal origin of the TICs observed in prophylactic specimens, these are likely primary lesions given the previously observed lack of direct implantation of tubal mucosa by ovarian endometrioid, mucinous and low-grade serous carcinomas (17, 196). A tubal origin is also supported by the observed residual risk (2-11%) of peritoneal carcinoma resembling ovarian cancer after prophylactic removal of the ovaries but not fallopian tubes (173, 174, 199, 200).

Altogether, recent studies of fallopian tubes and ovaries from BRCA1/2 mutation carriers and women with pelvic (fallopian tube, ovarian and peritoneal) SerCa suggest that, rather than arising de novo from the OSE at the ovarian surface as originally proposed, most SerCas likely arise from the FTE through a series of lesions, including p53 signatures, TICs and as yet unidentified intermediates.

1.13. Thesis hypothesis and objectives

Recent studies have strongly implicated the distal FTE as the source of high-grade SerCa, the most common and most lethal subtype of ovarian cancer. Most notably, analysis of prophylactic salpingectomy specimens from BRCA1/2 mutation carriers, at risk for tubal and ovarian SerCa, has consistently revealed occult carcinomas and putative histological cancer precursors in the FTE. This led to my overall hypothesis that molecular alterations involved in predisposition to both ovarian and fallopian tube SerCa would be evident in the non-malignant FTE of mutation carriers.
In the first study presented in this thesis (Chapter 2), gene expression profiles of microdissected non-malignant distal FTE [from BRCA mutation carriers (FTEb) and control patients (FTEn)] and ovarian and tubal SerCa were generated and compared. An equal number of FTEn and FTEb specimens were obtained during the luteal and follicular phase in light of the known impact of the ovarian cycle on the FTE. Importantly, this study demonstrated the presence of gene expression changes in FTEb which parallel changes observed in both tubal and ovarian SerCa. Furthermore, post-ovulatory FTEb luteal samples showed expression profiles that most resembled those of SerCa specimens, suggesting that the luteal phase milieu may contribute to serous carcinogenesis.

The second study in this thesis (Chapter 3) explored the hypothesis that differential relative expression of PR isoforms in FTEb compared to FTEn specimens may alter the response to luteal progesterone and contribute to an increased propensity for malignant transformation. While PRB was similarly expressed in all non-malignant FTE, luteal samples showed decreased nuclear and cytoplasmic immunopositivity for PRA compared to FTE obtained during the follicular phase. Although the resulting PRB predominance in luteal samples would likely influence gene expression, the finding of similar relative isoform expression in FTEn and FTEb samples suggests that altered response to a component of the luteal phase milieu other than progesterone may be involved in predisposition to SerCa.

In the third study of this thesis (Chapter 4), I investigated the possibility that FTEb respond differently to ovulation-associated inflammatory cytokines that are locally elevated during the luteal phase. Through IHC and gene-specific analysis of previously generated profiles, I demonstrate that FTEb specimens previously found to cluster with SerCa show evidence of increased NFκB-dependent (pro-inflammatory) signalling and diminished GR-dependent (anti-inflammatory) signalling. Similarly altered inflammatory gene expression profiles were also observed in SerCa specimens. Importantly, the results discussed in this chapter suggest a novel protective role for GR signalling against SerCa development.
The final study in this thesis (Chapter 5) explored the role of disabled homolog 2 (DAB2), an adaptor molecule decreased in SerCa and FTE luteal samples, in regulation of GR and NFκB signalling using an ovarian cancer cell model. Through transfection experiments I demonstrate that DAB2 promotes both GR transactivation and transrepression activity (likely in part via protein-protein interactions), resulting in decreased NFκB target gene expression. These findings suggest that decreased DAB2 may contribute to an altered inflammatory response in FTE luteal samples. Altogether, the work described in this thesis suggests that altered inflammatory signalling in FTE cells during the luteal phase is an important contributor to predisposition to (both tubal and ovarian) SerCa. As a result of these findings, I present a novel model for serous carcinogenesis which incorporates my data with known risk factors.
CHAPTER 2

GENE EXPRESSION PROFILES OF LUTEAL PHASE FALLOPIAN TUBE
EPITHELium FROM BRCA MUTATION CARRIERS RESEMBLE HIGH-GRADE
SEROUS CARCINOMA

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NOTES:
I completed all of the work presented in this chapter, with the exception of IHC which was performed by Kelvin So and BTSVQ clustering which was done by Dr. Igor Jurisica.
2.1: ABSTRACT

**Purpose:** To identify molecular alterations potentially involved in predisposition to adnexal SerCa in the non-malignant FTE of BRCA1/2 mutation carriers, given recent evidence implicating the distal FTE as a common source for SerCa.

**Experimental Design:** We obtained and compared gene expression profiles of laser capture microdissected non-malignant distal FTE from 12 known BRCA1/2 mutation carriers (FTEb) and 12 control women (FTEn) during the luteal and follicular phase, as well as 13 high-grade tubal and ovarian SerCa.

**Results:** Gene expression profiles of tubal and ovarian SerCa specimens were indistinguishable by unsupervised cluster analysis and significance analysis of microarrays. FTEb samples as a group, and four individual FTEb samples from the luteal phase in particular, clustered closely with SerCa rather than normal control FTE. Differentially expressed genes from these four samples relative to other FTEb samples, as well as differentially expressed genes in all FTEb luteal samples relative to follicular samples, were mapped to the I2D protein-protein interaction database, revealing a complex network affecting signaling pathways previously implicated in tumorigenesis. Two candidates, disabled homolog 2 mitogen-responsive phosphoprotein (DAB2) and Ski-like (SKIL), were further validated by real-time quantitative PCR (RT-qPCR) and tissue arrays. FTEb luteal and (both hereditary and sporadic) SerCa samples expressed higher levels of oncogenic SKIL and decreased levels of tumor suppressor DAB2, relative to FTEb follicular samples.

**Conclusions:** These findings support a common molecular pathway for adnexal SerCa and implicate factors associated with the luteal phase in predisposition to ovarian cancer in BRCA mutation carriers.
2.2: INTRODUCTION

High-grade SerCa is the most common histologic type of both ovarian and fallopian tube carcinoma, presents at an advanced stage of disease, and has a high mortality rate. Approximately 15% of SerCa cases are considered hereditary, arising in association with a germline mutation in the BRCA1 or BRCA2 genes (22, 23), which confer an estimated 50% to 60% or 18% to 23% lifetime risk, respectively (34, 201).

Until recently, no reproducible histologic cancer precursor of high-grade SerCa had been identified (15, 183, 184), but the unexpected finding of occult SerCa in the fallopian tubes of BRCA mutation carriers undergoing prophylactic surgery has led to the description of putative cancer precursors in the distal FTE (24, 191, 193, 194). These discoveries have led investigators to postulate that the distal FTE is an alternative source of SerCa designated as of ovarian origin by pathologists (24, 103, 180). Comparison of SerCa and coexisting TIC has shown identical mutations of the tumor suppressor TP53 in each lesion, suggesting that mutation of TP53, a frequent event in SerCa, occurs before the development of invasive disease and supports the idea that they are causally related (196). Although it is not possible to definitively prove the tubal origin of TICs, these are likely primary lesions given the previously observed resistance of tubal mucosa to direct implantation (17, 196). This concept is further supported by the finding that gene expression profiles of ovarian SerCa are more similar to normal FTE than the OSE (105), as well as by the similar risk factors (176, 182) and genomic alterations of ovarian and fallopian tube cancers (179, 202).

To identify molecular alterations that may be involved in predisposition to SerCa in the non-malignant FTE of BRCA1/2 mutation carriers, we generated and compared gene expression profiles of laser capture microdissected epithelial cells from the non-malignant distal fallopian tube of women at a baseline risk for SerCa (FTEn) and known BRCA1/2 mutation carriers (FTEb), as well as from high-grade tubal and ovarian SerCa specimens (fallopian tube SerCa and ovarian SerCa, respectively). To our knowledge, this is the only study to date of
gene expression profiles in the histologically normal FTE of *BRCA1/2* mutation carriers and control patients.

### 2.3: MATERIALS AND METHODS

#### 2.3.1. Study samples

The study was approved by the University Health Network Research Ethics Board, and all patients provided informed consent. Snap-frozen tissues were selected from the University Health Network Ovarian Tissue Bank and Database, including histologically normal fallopian tubes from 12 *BRCA* mutation carriers (FTEb) and from 12 control women undergoing salpingo-oophorectomy for reasons other than adnexal malignancy or family history (FTEn). Consequently, these women had not been tested for the presence of a *BRCA1/2* mutation. All women were pre-menopausal, and patients undergoing treatment for breast carcinoma, on hormonal therapy, or with a diagnosis of endometrial carcinoma were excluded. Although 3 of 12 mutation carriers had a history of breast carcinoma, they were not taking anti-estrogenic therapy at the time of surgery, with an average time since last treatment of 45.7 mo [95% confidence interval (95% CI), 6.6-84.7]. Non-malignant FTEn and FTEb specimens were age-matched, with an average age of 44.5y (95% CI, 42.3-46.7) for control patients and 41.3 y (95% CI, 39.4-43.2) for mutation carriers. Each group of FTE included six samples obtained during the luteal phase and six from the follicular phase of the ovarian cycle, determined after review of histologic sections of ovaries and endometrium by a gynecologic pathologist (P.A.S.), to account for potential effects of the endocrine milieu on gene expression.

Thirteen SerCa samples designated as of either tubal (n = 6) or ovarian (n = 7) origin from 11 patients were included. Two independent samples were obtained from two of the patients for direct comparison of tubal and ovarian SerCa; one of these patients did not have a known family history or *BRCA1/2* mutation. The remaining five ovarian SerCa patients had either a strong family history or identified mutation, whereas two of four remaining tubal SerCa
patients fulfilled these criteria. The other two of four tubal SerCa patients did not have a documented family history or identified mutation, but were selected based on a similar age of cancer onset as the ovarian cases. Overall, tubal and ovarian specimens were age-matched, with an average age of 54.8 y (95% CI, 49.4-60.3) and 49.6 y (95% CI, 42.6-56.5), respectively.

In addition, 12 of 13 of the SerCa specimens showed nuclear accumulation of p53 protein by IHC (not shown). Relevant clinical information on all study patients is summarized in Table 2.1. Ten nanograms of total RNA from study cases were reverse transcribed and linearly amplified using the NuGEN Biolin Ovation kit (NuGEN Technologies). Four tubal SerCa specimens were also selected based on a similar age of cancer onset as the ovarian cases. Overall, tubal and ovarian specimens were age-matched, with an average age of 54.8 y (95% CI, 49.4-60.3) and 49.6 y (95% CI, 42.6-56.5), respectively.

**2.3.2. Laser capture microdissection and RNA extraction**
Table 2.1. Relevant clinical data for samples used for gene expression profiling.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Age</th>
<th>Indication for Surgery/ Diagnosis</th>
<th>Cycle Status</th>
<th>BRCA1/2 Status</th>
<th>Family History</th>
<th>Previous Breast Cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Normal Control FTE (FTEa)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>46</td>
<td>history of cervical carcinoma</td>
<td>Luteal</td>
<td>NA</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>2</td>
<td>46</td>
<td>myometrial leiomyoma</td>
<td>Luteal</td>
<td>NA</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>3</td>
<td>42</td>
<td>grade 3 invasive squamous cell carcinoma of cervix; adenomyosis</td>
<td>Luteal</td>
<td>NA</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>4</td>
<td>36</td>
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<td>Luteal</td>
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<td>No</td>
</tr>
<tr>
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<td>43</td>
<td>left ovary serous cystadenoma; adenomyosis</td>
<td>Luteal</td>
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</tr>
<tr>
<td>6</td>
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<tr>
<td>7</td>
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<td>Follicular</td>
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</tr>
<tr>
<td>8</td>
<td>42</td>
<td>grade 1 invasive adenocarcinoma of cervix; adenomyosis</td>
<td>Follicular</td>
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<td>No</td>
</tr>
<tr>
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<td>Follicular</td>
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<td>No</td>
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<tr>
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<tr>
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<td>No</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BRCA1/2-mutated FTE (FTEb)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>44</td>
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<td>Luteal</td>
<td>BRCA1 5382insC</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>14</td>
<td>45</td>
<td>myometrial leiomyoma</td>
<td>Luteal</td>
<td>BRCA1 185delAG</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>15</td>
<td>38</td>
<td>NDH</td>
<td>Luteal</td>
<td>BRCA1 K1050T</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
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<td>40</td>
<td>leiomyoma</td>
<td>Luteal</td>
<td>BRCA1 185delAG</td>
<td>Unknown</td>
<td>No</td>
</tr>
<tr>
<td>17</td>
<td>39</td>
<td>NDH</td>
<td>Luteal</td>
<td>BRCA1 185delAG</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>18</td>
<td>46</td>
<td>right ovary cortical epithelial inclusions with hyperplasia and serous tubal metaplasia; left ovary serous cyst</td>
<td>Luteal</td>
<td>BRCA2 G5810G</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>19</td>
<td>42</td>
<td>myometrium leiomyoma</td>
<td>Follicular</td>
<td>BRCA1 5382insC</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>20</td>
<td>40</td>
<td>right ovary mature cystic teratoma negative for malignancy</td>
<td>Follicular</td>
<td>BRCA1 185delAG</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>21</td>
<td>47</td>
<td>leiomyomata</td>
<td>Follicular</td>
<td>BRCA2 6174delT</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>22</td>
<td>39</td>
<td>bilateral ovaries cystic follicles negative for malignancy</td>
<td>Follicular</td>
<td>BRCA1 1293del40</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>23</td>
<td>37</td>
<td>NDH</td>
<td>Follicular</td>
<td>BRCA1 185delAG</td>
<td>Unknown</td>
<td>Yes</td>
</tr>
<tr>
<td>24</td>
<td>39</td>
<td>NDH</td>
<td>Follicular</td>
<td>BRCA1 185delAG</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fallopian tube SerCa</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>41</td>
<td>right tube poorly differentiated carcinoma favor serous type; grade 3; bilateral ovaries negative for malignancy</td>
<td>NA</td>
<td>Negative</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>25†</td>
<td>57</td>
<td>left tube grade 3 serous carcinoma; right tube intramucosal carcinoma; bilateral ovaries grade 3 serous carcinoma</td>
<td>NA</td>
<td>Unknown</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Sample</td>
<td>Age</td>
<td>Indication for Surgery/Diagnosis</td>
<td>Cycle Status</td>
<td>BRCA1/2 Status</td>
<td>Family History</td>
<td>Previous Breast Cancer</td>
</tr>
<tr>
<td>--------</td>
<td>-----</td>
<td>-------------------------------------------------------------------------------------------------</td>
<td>--------------</td>
<td>----------------</td>
<td>----------------</td>
<td>-----------------------</td>
</tr>
<tr>
<td>27‡</td>
<td>57</td>
<td>right tube grade 3 serous carcinoma; bilateral surface ovarian grade 3 serous carcinoma</td>
<td>NA</td>
<td>Unknown</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>28</td>
<td>59</td>
<td>right tube grade 3 serous carcinoma, metastasis to left tubal serosa and ovarian surface; right ovary NDH</td>
<td>NA</td>
<td>Unknown</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>29</td>
<td>57</td>
<td>right tube grade 3 serous carcinoma, metastasis to right ovary; left ovary primary serous carcinoma</td>
<td>NA</td>
<td>Unknown</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>30</td>
<td>58</td>
<td>left tube grade 3 serous carcinoma; right tube mucosal epithelial hyperplasia; bilateral ovaries grade 3 serous carcinoma</td>
<td>NA</td>
<td>BRCA1</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

**Ovarian SerCa**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Age</th>
<th>Indication for Surgery/Diagnosis</th>
<th>Cycle Status</th>
<th>BRCA1/2 Status</th>
<th>Family History</th>
<th>Previous Breast Cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>31</td>
<td>54</td>
<td>bilateral ovaries grade 3 transitional cell carcinoma with focal serous differentiation</td>
<td>NA</td>
<td>BRCA1</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>32</td>
<td>55</td>
<td>ovarian grade 3 papillary serous carcinoma</td>
<td>NA</td>
<td>Unknown</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>33</td>
<td>50</td>
<td>bilateral ovarian grade 3 papillary serous carcinoma, extension through capsule into right fallopian tube; left tube NDH</td>
<td>NA</td>
<td>BRCA1 1917delITT</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>34</td>
<td>42</td>
<td>bilateral ovaries and tubes grade 3 serous carcinoma</td>
<td>NA</td>
<td>BRCA1 3875delGCT</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>35‡</td>
<td>57</td>
<td>see 26</td>
<td>NA</td>
<td>Unknown</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>36‡</td>
<td>57</td>
<td>see 27</td>
<td>NA</td>
<td>Unknown</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>37‡</td>
<td>52</td>
<td>bilateral ovaries and tubes grade 3 serous carcinoma</td>
<td>NA</td>
<td>BRCA1</td>
<td>Yes</td>
<td>No</td>
</tr>
</tbody>
</table>

NDH refers to no diagnostic histopathology observed at pathologic review. NA represents data points that are not applicable/not evaluated at the time of case selection. ‡ represents two independent carcinoma samples obtained from the same patient, as does †.
provide sufficient material for global gene expression analysis from limited total RNA, while accurately and reproducibly maintaining differential transcript abundance in non-amplified samples (203). Briefly, first-strand cDNA synthesis was done using a unique DNA/RNA primer, followed by second strand cDNA synthesis and SPIA isothermal linear amplification. Amplified cDNA was then purified, enzymatically fragmented, and labeled with biotin. Quality and quantity of the purified labeled cDNA product were confirmed before hybridization to Affymetrix GeneChip U133A Plus 2.0 arrays (Affymetrix, Inc.) according to manufacturer’s instructions (protocols available at http://www.microarrays.ca/). Amplification and hybridization of samples was done in six separate runs, with each group represented in each run (FTEn from luteal and follicular phase, FTEb from luteal and follicular phase and tubal and ovarian SerCa). Profiling data for all samples has been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) and are accessible through Gene Expression Omnibus Series accession number GSE10971.

2.3.4. **Statistical analysis of gene expression profiles**

All CEL files were imported into ArrayAssist (Stratagene), and a master table of all hybridizations was created using the robust multiarray average (RMA) algorithm. Acceptable array performance was confirmed by review of generated RPT files. RMA normalized data were then used as the basis for all subsequent clustering and statistical analysis. Unsupervised binary tree-structured vector quantization clustering (BTSVQ) was done using all SerCa samples. This method combines tree-structured vector quantization and partitive k-means clustering to separate samples into homogenous groups based on similar levels of gene expression (204). ArrayAssist was used to perform unsupervised hierarchical clustering of all SerCa cases following definition of tubal SerCa as baseline. Hierarchical clustering was subsequently done on all hybridizations following definition of FTEn samples as baseline, as well as on data grouped by major class (FTEn, FTEb, or SerCa) or subgroup (FTEn luteal,
FTEn follicular, FTEb luteal, FTEb follicular, fallopian tube SerCa, ovarian SerCa) after defining FTEn and FTEn luteal as baseline, respectively.

RMA normalized data were then imported into Microsoft Excel for identification of differentially expressed genes using significance analysis of microarrays (SAM version 3.0, available at http://www-stat.stanford.edu/~tibs/SAM/). For each comparison, two-class unpaired analysis was done using unlogged data, and the delta value was selected based on an acceptable corresponding median false discovery rate (FDR). To find overlapping significant probe sets, relevant significance lists were imported into ArrayAssist and compared using the Venn diagram function. Additional information for probe sets of interest, including gene ontology, was obtained using the batch query function on the NetAffx Analysis Center portion of the Affymetrix Web site (http://www.affymetrix.com/analysis/index.affx).

2.3.5. Identification of candidate genes using protein-protein interaction networks

To visualize the interactive networks of genes found significantly altered, as well as to aid in the selection of the most promising candidates for follow-up studies, a combination of bioinformatic tools was used. Lists of significant probe set IDs separated by analysis and direction of change in the group of interest were submitted to the I2D (Interlogous Interaction Database) Web site version 1.6 (http://ophid.utoronto.ca/i2d) to obtain first-degree interaction information for the corresponding proteins (205). Next, known and predicted protein-protein interactions for each list were visualized using NAViGaTOR (Network Analysis, Visualization, and Graphing TORonto) software, version 1.1.0, to elucidate the connectivity of the significantly altered genes (http://ophid.utoronto.ca/navigator). The extent of overlap of individual networks of interest was visualized using the Compare Networks (union) option. Proteins of extreme interest (p53 and BRCA1) were then located by Swiss Prot ID, and all non-interacting proteins were deleted.
2.3.6. RT-qPCR

Three representative samples from each nonmalignant group (FTE\textsubscript{n} luteal, FTE\textsubscript{n} follicular, FTE\textsubscript{b} luteal, FTE\textsubscript{b} follicular) and six SerCa samples were selected for validation of gene expression profiles. Ten nanograms of RNA were linear amplified using the NuGEN Ovation RNA Amplification System (NuGEN Technologies). RNA was also isolated from SKOV3 ovarian cancer cells using TRIzol reagent (Invitrogen Corporation) for use as a calibrator sample. Contaminating DNA was removed (DNA-free kit, Ambion, Inc.), and first-strand cDNA was generated using SuperScript III Reverse Transcriptase and Oligo(dT)\textsubscript{20} primers (Invitrogen). Quality and quantity of all amplified cDNA were confirmed, and all samples were diluted to 1.6 ng/µL using sterile double distilled water.

Primer Express Software version 2.0 (Applied Biosystems) was used to select specific primers for human disabled homolog 2 mitogen-responsive phosphoprotein (DAB2), Ski-like (SKIL), and reference gene b-actin (ACTB) based on the sequence of the relevant probe set(s). All amplicons were situated within the first 1,500 bp from the poly(A) tail, given the use of oligo(dT)-primed reverse transcription of study and calibrator samples. Selected primer sequences included DAB2 forward 5'-CTAGCTATTGCAAATGAGGGAAG-3', DAB2 reverse 5'-GGTAATACTACTTGAAACCAGGAGCA-3', SKIL forward 5'-AACTGTCTCTACCTCAGTGTG-3', SKIL reverse 5'-AAGCTCAGAGCAGCTATGATG-3', ACTB forward 5'-GCATTGGTTACAGGAAGTCCCTTG-3', and ACTB reverse 5'-CTATCACCTCCCCCTGTTGGA-3'. Endpoint PCR was done for all primer sets, and the reaction product was sequence verified.

RT-qPCR was done using the QuantiTect SYBR Green PCR kit (Qiagen, Inc.) according to manufacturer’s instructions. PCR was done using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems), with use of the ROX internal reference dye. A dissociation reaction was done at the end of the PCR program to confirm amplification of a single product at the expected melting temperature, and the product was run on a 2.5% agarose gel to confirm correct size. All experiments included triplicate wells of each sample for both target and reference gene. The comparative CT method for relative quantitation was done...
using ABI PRISM Sequence Detection Software version 2.0 (Applied Biosystems). Target gene CT values were normalized to ACTB. Statistical analysis was done using one-way ANOVA followed by a least significant difference test for post hoc comparisons (p < 0.05).

2.3.7. IHC

A tissue microarray consisting of a larger subset of non-malignant fallopian tube specimens from control patients and mutation carriers was constructed using the semi-automated TMArrayer (Pathology Devices, Inc.). Patient samples were identified according to history and diagnosis from the University Health Network Ovarian Tissue Bank and Database. Routine H&E sections were examined and a 1.5mm² area of histologically normal FTE was selected before transfer of the corresponding formalin-fixed paraffin-embedded tissue core to the master block. The final array consisted of a total of 33 non-malignant specimens from confirmed BRCA1/2 mutation carriers (including 11 luteal, 16 follicular, 3 early follicular, and 3 perimenopausal), 25 normal control specimens (including 11 luteal, 12 follicular, and 2 perimenopausal), and 2 cores each of normal kidney and liver for orientation purposes. All non-malignant FTEn and FTEb cases used for gene expression profiling were included. Duplicate 5 µm sections of the array were included on each slide to control for potential variability during IHC. In addition, a separate array containing duplicate 0.6 mm² cores obtained from 59 cases of high-grade SerCa was also used to assess the expression of candidates in both hereditary and sporadic carcinoma tissue specimens (n=24 cases associated with known family history, n=23 cases not associated with family history, n=12 unknown; average age, 61.8 y; 95% CI, 59.1-64.5). All but 3 carcinoma specimens were independent from those subjected to gene expression profiling, and all cases were obtained from the Ovarian Tissue Bank and Database and were naive to chemotherapy.

IHC was done using standard procedures following microwave antigen retrieval using goat polyclonal anti-SKIL antibody [SnoN (K-20), Santa Cruz Biotechnology] at a dilution of 1 in
600, mouse monoclonal anti-DAB2 (disabled-2/p96 antibody, BD Biosciences) at a dilution of 1 in 50, or mouse monoclonal anti-cdc2 [cdc2 (P0H1), Cell Signaling Technology, Inc.] at a dilution of 1 in 50. Antibody concentrations were optimized on normal or malignant breast tissue sections before staining tissue array slides.

After IHC, the ScanScope CS slide scanner (Aperio Technologies, Inc.) was used to create digital slide images at 20X magnification. Tissue cores were then visualized and manually scored using ImageScope software (Aperio Technologies), version 6.25. Scoring was independently done by two observers (A.A.T. and P.A.S.) blinded to patient information. Scores were given for percentage of epithelial cells stained (0, 0%; 1, 1-24%; 2, 25-50%; 3, >50%), as well as intensity of staining (0, negative; 1, light; 2, medium; 3, dark). The combined histoscore was obtained by adding these two individual scores, and nuclear and cytoplasmic staining was reviewed independently (206, 207). Statistical analysis was done using ANOVA followed by least significant difference (p < 0.05). Comparison of unpaired proportions was by Fisher’s exact test (p < 0.05).

2.4: RESULTS AND DISCUSSION

2.4.1. High-grade ovarian SerCa and fallopian tube SerCa exhibit indistinguishable gene expression profiles

An initial critical question was whether SerCa designated as of ovarian origin, according to current pathology practice, is molecularly similar to that apparently derived from the fallopian tube. A high degree of similarity would support the idea that the FTE is the cell of origin for both tubal and ovarian SerCa. Unsupervised hierarchical (agglomerative) clustering of the individual SerCa samples after RMA normalization showed that the tubal and ovarian specimens had closely related global gene expression profiles (Figure 2.1A). SerCa of presumed ovarian origin clustered together, with many of the tubal cancers clustering more closely with the ovarian cancers than with other tubal cancers. To examine this relationship more closely, an
A.

B.

C. Decreased in SerCa

\[931 \quad 2235 \quad 1097\]

SerCa FT vs. FTEn (3166)
SerCa Ov vs. FTEn (3332)

Increased in SerCa

\[1112 \quad 3267 \quad 2912\]

SerCa FT vs. FTEn (4399)
SerCa Ov vs. FTEn (6199)
FIGURE 2.1: Comparison of fallopian tube and ovarian SerCas. Unsupervised hierarchical (agglomerative, $A$) and BTSVQ (partitive, $B$) clustering was done using all grade 3 SerCas, revealing the similarity of samples of presumed tubal and ovarian origin. Numbers shown at terminal ends in $A$ and $B$ represent sample numbers, as indicated in Table 2.1. $A$, FT, fallopian tube carcinomas; $Ov$, ovarian carcinomas. $B$, ovarian and tubal cancers associated with a known $BRCA1/2$ mutation and/or family history are represented by open and closed bars, respectively. Ovarian and tubal cancers with unknown familial status are represented by hatched and spotted bars, respectively. $C$, Venn diagrams were used to compare probe sets differentially expressed by SAM between FTE from normal controls and tubal and ovarian carcinomas. At an FDR of 4.6%, 5,522 of 11,574 of the total probe sets (48%) were found to be altered in tumors of both tubal and ovarian origin ($boxed$).
unsupervised partitive clustering method, BTSVQ, was used. This method iteratively partitioned the 13 cases by a k-means algorithm (k=2), considering the partitioning of all probe set responses by a self-organizing map algorithm. This method clearly revealed that samples partitioned together irrespective of presumed origin or known mutation status (Figure 2.1B). Although similar, identical results were not obtained using the two clustering methods. This is likely due to the distinct approaches (agglomerative versus partitive), as well as the differential sensitivity of each method to data normalization (204). Most notably, while matched tubal and ovarian SerCa specimens 26 and 35 grouped closely together using BTSVQ clustering (Figure 2.1B), this was not observed using hierarchical cluster analysis (Figure 2.1A). In contrast, paired tumours 27 and 36 clustered closely together using both methods. This discrepancy could potentially be explained by the lower quality of sample 26 relative to all other SerCa specimens (data not shown), which may have a greater impact on results achieved through hierarchical vs. BTSVQ analysis. Two-class paired SAM analysis of the two tubal and ovarian cancers from the same patients revealed no differentially expressed genes at a minimum FDR of 40%, as did two-class unpaired analysis of the remaining carcinoma specimens at an FDR of 21%. Thus, cluster analysis, as well as SAM, indicates that SerCas have similar molecular profiles whether of presumed ovarian or tubal origin.

In contrast, comparison of all FTEn samples with all cancers revealed 4,263 probe sets with decreased expression and 7,311 probe sets with increased expression in the cancers at an FDR of 4.3%. Of the down-regulated probe sets, 2,235 (52%) were shared between the two malignancies, as were 3,287 (45%) of the up-regulated probe sets (Figure 2.1C). Given the high degree of similarity between fallopian tube and ovarian SerCa, all subsequent comparisons to malignant samples were done combining the SerCas as a single group.
2.4.2. Differential expression of genes in a subset of histologically normal FTEb samples

Hierarchical clustering using all probe sets was done to determine the overall similarity of FTEb and SerCa samples. Analysis using grouped data revealed that FTEb samples, which were histologically indistinguishable from FTEn, clustered with SerCa samples rather than normal controls (Figure 2.2A), demonstrating that gene expression altered in mutation carriers is consistent with changes that have occurred in malignant cells. These changes likely reflect and contribute to the overall increased risk for malignant transformation in mutation carriers, consistent with the documented roles of BRCA1 and BRCA2 proteins in maintaining genomic integrity through participation in DNA repair, transcriptional regulation, and cell cycle control (39, 43, 44).

Unsupervised clustering using individual data revealed that four BRCA1-mutated FTEb samples clustered with all of the carcinomas, indicating that global gene expression in this subset of samples most closely resembles that of malignant epithelial cells (Figure 2.2B). Whereas it is not possible to determine if these cases would have been more likely to progress to SerCa had prophylactic surgery not occurred, it is feasible that these samples have acquired gene expression changes involved in serous carcinogenesis that have not yet resulted in atypical histology. SAM two-class unpaired analysis identified 288 probe sets with decreased expression and 598 with increased expression in these four samples relative to the remaining FTEb samples that were more similar to normal controls at an FDR of 4.3%. Gene ontology analysis revealed that many differentially expressed probe sets correspond to genes with known roles in BRCA1/2-dependent processes (transcriptional regulation, cell cycle control, ubiquitin cycle) (39, 44), as well as others involved in tumor initiation and progression in general (apoptosis, cell adhesion, and cell motility; Figure 2.2C; see Appendix I for a complete list of significantly altered genes arranged by gene ontology). In addition, 181 of 288 (63%) of the probe sets with decreased expression and 172 of 598 (29%) with increased expression overlapped in the same direction as those differentially expressed between non-malignant
FIGURE 2.2: Identification of differentially expressed genes in histologically normal FTEb. Hierarchical clustering was done using grouped data (A), revealing the overall similarity of FTEb and SerCa specimens. Unsupervised clustering was then done using individual hybridizations (B) to identify specific FTEb samples more closely resembling SerCa specimens compared with normal controls (boxed). Numbers shown at terminal ends in A and B represent sample numbers as indicated in Table 2.1. B, FTEn samples obtained during the luteal and follicular phase (grey and light blue, respectively), FTEb luteal and follicular samples (red and dark blue, respectively), and tubal and ovarian SerCa samples (green and black, respectively). SAM revealed the decreased expression of 288 probe sets and increased expression of 598 probe sets between these samples and the remaining FTEb specimens grouping with normal controls at 4.3% FDR [average fold-change of 2.9 (95% CI, 2.5-3.2) for decreased probe sets and 3.7 (95% CI, 3.4-4.0) for increased]. The distribution of primary ontologies of the genes represented by these probe sets is shown in (C). Venn diagrams were used to compare significantly altered probe sets to those differentially expressed between SerCa and FTEn specimens at the same FDR (D). Overlapping genes (boxed).
FTEn and SerCa samples as a group at the same FDR (4.3%), further supporting the idea that these may include some of the earliest events in serous carcinogenesis (Figure 2.2D).

2.4.3. Differential effect of the ovarian cycle in BRCA1/2 mutation carriers

Given the unexpected (in light of the proposed protective effect of progesterone) finding that all four FTEb samples grouping with SerCa were obtained during the luteal phase, we further explored the association of the ovarian cycle with FTE gene expression. Hierarchical clustering of all samples using all probe sets was repeated using data grouped by both BRCA mutation and ovarian cycle status. The dendrogram obtained confirmed that FTEb samples from the luteal phase (FTEb luteal) more closely resembled SerCa samples compared with FTEb samples from the follicular phase (FTEb follicular; Figure 2.3A).

Ovarian steroids are known to affect the growth and differentiation of the FTE. Estrogens act during the follicular phase to promote ciliogenesis and secretory cell hypertrophy, whereas progesterone, which is predominant during the luteal phase, induces dedifferentiation (169, 208). However, these hormonal influences are not widespread within the tube and can be quite variable among individuals; thus, both ciliated and secretory cells are present in the FTE throughout the cycle. Interestingly, SAM two-class unpaired analysis revealed that the number of differentially expressed probe sets between the luteal and follicular phases was far greater in mutation carriers than in normal controls. At an FDR of 9.8%, only 21 probe sets were found to be decreased in the luteal relative to the follicular phase in normal control patients (Figure 2.3B). In contrast, 322 probe sets were decreased and 70 were increased in the luteal relative to follicular phase in FTEb at the same FDR, suggesting that hormonal influences on the FTE may be altered as a result of reduced functional BRCA levels. Accordingly, the average BRCA1 level (as determined by directed gene-specific analysis of RMA-normalized expression profiles) in FTEb luteal samples was 50% lower than that seen in FTEb follicular samples, and an 80% decrease was observed in the four FTEb luteal samples clustering with SerCa relative to the remaining FTEb specimens (not shown). Previous studies
A.

- FTEn Luteal (1-6)
- FTEn Follicular (7-12)
- FTEb Follicular (19-24)
- FTEb Luteal (13-18)
- SerCa Ovarian (31-37)
- SerCa Tubal (25-30)

B.

- FTEn Decreased in Luteal (21)
- FTEb Decreased in Luteal (322)
- FTEn Increased in Luteal (0)
- FTEb Increased in Luteal (70)

- 18
- 3
- 319
- 0
- 0
- 70

C.

- Gene Ontology: Unknown, Regulation of Transcription, Signal Transduction, Immune Response, Cell Cycle, Protein Modification, Apoptosis, Cell Adhesion, Protein Transport, Proteolysis, Steroid Biosynthesis & Metabolism, Microtubule-Based Movement, Other

D.

- Decreased in Luteal FTEb only (319)
- Decreased in SerCa vs. FTEn (6381)
- Increased in Luteal FTEb only (70)
- Increased in SerCa vs. FTEn (8811)

- 205
- 114
- 6267
- 44
- 26
- 8785
FIGURE 2.3: The effect of the ovarian cycle on FTE gene expression in BRCA1/2 mutation carriers and normal controls. Hierarchical clustering using data grouped by mutation status and stage of the ovarian cycle at the time of surgery (A) confirmed the similarity of FTEb luteal and SerCa samples. SAM identified a greatly increased number of differentially expressed probe sets by ovarian cycle in mutation carriers compared with normal controls at an FDR of 9.8% (B). All differentially expressed probe sets in the normal controls were increased in the follicular phase, at an average fold-change of 2.7 (95% CI, 2.2-3.3) compared with luteal samples. Of the 392 differentially expressed probe sets in mutation carriers, 322 were increased in the follicular phase at an average fold-change of 2.7 (95% CI, 2.4-2.9) and 70 were increased in the luteal phase at an average fold-change of 3.4 (95% CI, 2.9-3.9) relative to follicular samples. The distribution of primary ontologies of the genes represented by the probe sets differentially expressed in mutation carriers but not normal controls (C). Venn diagrams were used to compare probe sets significantly altered in FTEb luteal samples to those differentially expressed between SerCa and FTEn specimens at an FDR of 8.3% (D).
have implicated both BRCA1 and BRCA2 in the regulation of gonadal steroid hormone receptor activity (32, 53), and this regulation may contribute to the differences in gene expression between control patients and BRCA1/2 mutation carriers.

The 389 probe sets specific to mutation carriers were subjected to gene ontology analysis and were found to correspond to BRCA1/2-dependent and tumorigenesis-involved processes similar to that observed in the previous analysis (Figure 2.3C; see Appendix II for a complete list of significantly altered genes arranged by gene ontology). Interestingly, 114 of 319 (36%) of the probe sets with decreased expression and 26 of 70 (37%) with increased expression in FTEb luteal samples overlapped in the same direction with probe sets differentially expressed between FTEn and SerCa samples at a similar FDR (8.3%; Figure 2.3D). This is in contrast to an overlap of 9 of 70 (13%) with decreased expression ($p = 0.0001$) and 59 of 319 (18%) with increased expression ($p = 0.001$) in FTEb follicular samples. That the profiles obtained during the luteal phase of mutation carriers most closely resembles the gene expression pattern of SerCa raises the possibility that the hormonal milieu associated with the luteal phase may contribute to cancer predisposition in some individuals, such as BRCA1/2 mutation carriers.

A primary hormonal difference between the follicular and luteal phases is the elevation of circulating progesterone during the luteal phase. Studies have frequently suggested that progesterone is a protective rather than exacerbating factor in ovarian cancer development (69, 108). Epidemiologic data generally show that women with conditions in which progesterone is elevated have a lower incidence of ovarian cancer. For instance, the protective effect of increased parity and twin pregnancies has been attributed to the greatly increased level of maternal circulating progesterone (101, 108, 176, 182). In addition, many studies have linked the use of combination oral contraceptives to a decreased ovarian cancer risk in mutation carriers and the general population. A few studies have further suggested that formulations with high progestin potency offer greater protection than those with low progestin potency (26, 32, 108, 112). Finally, treatment of normal OSE and ovarian cancer cells with progesterone has a
growth inhibitory effect (101, 108). Accordingly, some authors have suggested that exposure to progesterone may eliminate cells genetically damaged by incessant ovulatory events through induction of apoptosis, thus providing an exfoliation effect (101, 108). Evidence has emerged, however, that the effects of progesterone may be more complex than previously appreciated. For instance, whereas high concentrations of progesterone suppressed tumourigenesis in nude mice inoculated with ovarian cancer cells (209), lower concentrations increased the proliferative capacity of OSE and ovarian cancer cells in culture (109). This is consistent with the postulate that luteal-phase levels of progesterone have a growth promotional effect, whereas the higher levels achieved during pregnancy or OCP use induce cell cycle arrest or apoptosis of epithelial cells (108). Similar studies have not yet been reported for FTE cells. However, it is possible that exposure to luteal-phase levels of progesterone may not be protective against malignant transformation of the epithelial cells of the distal fallopian tube, similar to the effect observed in the breast (210-212), particularly in cells in which the DNA repair pathways may be compromised. It is of course possible that other hormonal changes associated with the luteal phase may affect gene expression in the FTE, and further studies are required using FTE cells in culture. Finally, while all non-malignant cases in this study have been age-matched, there is evidence that mutation carriers initiate menopause at an earlier age than normal controls (213), suggesting that the nature of the hormonal milieu of the luteal phase may be different in the two populations.

2.4.4. Identification of protein-protein interaction networks potentially involved in initiation of SerCa

To select genes for validation and further investigation, we queried the I2D protein interaction database with our set of differentially expressed genes from both SAM analyses. Of the 886 probe sets differentially expressed in the four FTEb sample grouping with SerCa compared with the remaining FTEb, 417 (47%) mapped to I2D, as well as 218 of 389 (56%) of the probe sets differentially expressed in FTEb luteal compared with FTEb follicular samples.
Because of the known involvement of p53 in high-grade SerCa, we focused our attention on differentially expressed genes encoding for proteins predicted to directly or indirectly interact with BRCA1/2 or p53 proteins. Because mutation of \( \text{BRCA1} \) or \( \text{BRCA2} \) leads to activation of the damage response pathway, loss of this pathway by somatic mutation of p53 and presumably other members is required for malignant transformation (20). Consequently, accumulation of p53 protein is frequently observed in the histologically normal FTE of mutation carriers and control patients, and decreased expression of cell cycle arrest genes p21 and p27 have been observed in prophylactic tubal specimens relative to normal controls (180, 191).

Networks obtained through I2D were visualized with NAViGaTOR software. One gene found in networks of both comparisons, DAB2, was found to be decreased in FTEb luteal and SerCa samples. DAB2 is an adaptor molecule that exerts its tumor suppressive function largely through its role in facilitating TGFβ-induced growth inhibition (214), a pathway that must be circumvented in early stages of epithelial carcinogenesis and which has previously been shown to be dysfunctional in ovarian cancer (215). In addition, we selected SKIL, which was upregulated in FTEb luteal samples and acts to inhibit Smad2 and Smad3 (216), targets that are enhanced by DAB2 (Figure 2.4). The implication of expression changes in the TGFβ pathway regulatory genes DAB2 and SKIL in FTEb samples during the luteal phase is highlighted by the observation that 15% of the significantly altered probe sets found by both SAM analyses (185 of 1,201 unique probe sets) correspond to genes known to be downstream TGFβ targets (217-220), consistent with an altered TGFβ response (see Appendices I+II). Such genes include DAB2 (217, 219) and SKIL (218, 219) themselves, in addition to well-established TGFβ targets ID2, ETS2, and PIM1 (220).
Altered expression of TGF-β target genes
(185 probe sets differentially expressed)
FIGURE 2.4: Protein-protein interaction subnetwork potentially involved in initiation of SerCa. Submission of probe sets differentially expressed in the FTEb specimens that clustered with SerCa, as well as those specifically altered in FTEb luteal samples to the online Interlogous Interaction Database (I2D, version 1.6), revealed overlapping networks of proteins with altered expression in the four FTEb samples and/or FTEb luteal samples overall. The interesting subnetwork containing the TGFβ pathway regulators DAB2 and SKIL is expanded here. Genes encoding proteins shown as upward-pointing triangles were increased in FTEb luteal samples, whereas those shown as downward-pointing triangles were decreased in the same samples. Known and predicted interactions between proteins are indicated by a line, with activation represented by an arrowhead and inhibition represented by a blunt end. *TGFβ target genes identified by select gene expression profiling studies (217-220).
2.4.5. Differential expression of SKIL and DAB2 in FTEb samples during the luteal phase

In agreement with our Affymetrix data, FTEb luteal samples were found to express higher levels of SKIL mRNA compared with FTEb follicular samples as determined by RT-qPCR, although this did not reach statistical significance (Figure 2.5A, left). A further increase in SKIL mRNA was observed in SerCa samples compared with FTEb follicular (p < 0.05). IHC on tissue microarrays revealed a similar average intensity of nuclear staining in FTEb luteal and SerCa samples, as well as a trend toward increased staining in FTEb luteal compared with FTEb follicular samples (Figure 2.5A, right). Representative images of SKIL immunohistochemical staining are shown in Figure 2.5B. Also consistent with our profiling data, FTEb luteal samples expressed a level of DAB2 mRNA similar to SerCa samples and a greatly reduced expression compared with FTEb follicular samples (p < 0.05; Figure 2.5C, left). IHC for DAB2 on tissue microarrays revealed that FTEb luteal and SerCa samples express a decreased level of cytoplasmic DAB2 protein compared with FTEb follicular samples (p < 0.05; Figure 2.5C, right). A lower proportion of FTEb luteal (one of nine, 11%) and SerCa (1 of 48, 2%) samples had a histoscore of >4 compared with FTEb follicular specimens (13 of 16, 81%). The decrease in DAB2 during the luteal phase could partly be explained by altered cdc2 protein levels. Previous studies have indicated that functional BRCA1 indirectly inhibits cdc2 kinase activity (221), which then negatively regulates DAB2 through cell cycle-specific phosphorylation (222). Absence of functional BRCA proteins would therefore lead to accumulation of active cdc2 and subsequent reduction of DAB2. Accordingly, in direct opposition to DAB2, there was a trend toward an increased nuclear histoscore for cdc2 in FTEb luteal compared with FTEb follicular samples, and a similar proportion of FTEb luteal (4 of 11, 36%) and SerCa (18 of 51, 35%) samples showed intense nuclear staining. Representative images of DAB2 and cdc2 immunohistochemical staining are shown in Figure 2.5D. Most intriguingly, whereas FTEb luteal and SerCa samples showed an overall loss of cytoplasmic DAB2 staining, FTEb follicular samples typically showed retention of staining in the secretory cells of the mucosal lining.
FIGURE 2.5: Expression of SKIL, DAB2, and cdc2 in FTEb luteal and SerCa samples. To confirm the increased expression of SKIL and decreased expression of DAB2 in FTEb luteal and SerCa samples, RT-qPCR was done using cDNA generated from a subset of samples previously used for gene expression profiling (n = 3 for each nonmalignant group, as well as six SerCa). IHC was also done on tissue microarrays containing a total of 58 non-malignant FTE from BRCA1/2 mutation carriers and normal controls from both phases of the ovarian cycle, as well as 59 grade 3 SerCas. RT-qPCR (A, left) and IHC on tissue microarrays (A, right) confirmed a trend toward increased average expression of SKIL in FTEb luteal and SerCa samples compared with FTEb follicular and normal controls (images shown in B, magnification 40x). RT-qPCR (C, left) and IHC (C, right) also confirmed the decreased average expression of DAB2 in FTEb luteal and SerCa samples compared with FTEb follicular specimens (images shown in D, top; magnification 40x). Specific retention of DAB2 staining in the secretory (solid arrow) compared with ciliated (dashed arrow) cells of the mucosal lining in FTEb follicular samples is highlighted. This differential pattern of staining was also observed in normal control patients, although the difference between the histoscore of FTEn luteal and FTEn follicular samples did not attain statistical significance. Similar to SKIL and in contrast to DAB2, cdc2 protein showed increased nuclear intensity in FTEb luteal (n=11) and SerCa (n=51) samples compared with FTEb follicular (n=16) specimens (images shown in D, bottom; magnification 40x). FTEb luteal samples had an increased average nuclear histoscore compared with FTEb follicular samples, although this did not attain statistical significance (4.91; 95% CI, 4.50-5.32 versus 4.20; 95% CI, 3.72-4.68). No difference in staining was observed in luteal (n=11) and follicular (n=12) samples from normal controls (data not shown). Vertical bars shown in A and C represent average mRNA or protein expression for each group of samples, with statistically significant differences in average mRNA or protein expression indicated by different letters (one-way ANOVA with least significant difference post hoc test; p < 0.05). Black circles in A and C indicate the individual sample values for RT-qPCR.
resulting in an increased overall intensity and histoscore. The specific loss of DAB2 expression in secretory cells during the luteal phase may explain the modest difference in mRNA and protein expression between luteal and follicular samples when including both secretory and ciliated cell populations and further emphasizes the importance of this cell type in serous carcinogenesis (17, 194).

Many studies have observed a similar loss of DAB2 in ovarian carcinomas compared with normal OSE (223-225), although this has not previously been found in the histologically normal OSE from BRCA1/2 mutation carriers. Interestingly, mice heterozygous for DAB2 frequently develop epithelial dysplasia on the ovarian surface but not malignant ovarian tumors, suggesting that loss of DAB2 is necessary but not sufficient for ovarian carcinoma development (223); however, the oviductal epithelium was not examined. Whereas functional DAB2 exerts its tumor-suppressive effect by mediating TGFβ-induced growth inhibition through transmission of signals from the TGFβ receptors to the Smad transcriptional activators (214), this is opposed by SKIL, which directly represses Smad gene transcription and enhances cytoplasmic sequestration of active Smad protein complexes (216, 226, 227). Although SKIL has not previously been implicated in ovarian cancer development, it lies within a chromosomal region (3q26) previously found to be amplified in a majority of serous fallopian tube and ovarian carcinomas by comparative genomic hybridization (179). SKIL protein is highly elevated in many human cancer cell lines, including ovarian, and has been found to promote tumor growth in nude mice (228). High expression of SKIL protein is also a negative prognostic factor in ER+ breast carcinomas (226). It is therefore likely that the combined effect of decreased DAB2 and increased SKIL in FTEb would promote malignant transformation through potent suppression of TGFβ-induced cell cycle arrest and apoptosis.

In conclusion, gene expression changes potentially involved in the earliest events of tubal and ovarian SerCa have been identified in histologically normal FTE from BRCA1/2 mutation carriers. These expression changes seem to be influenced by reproductive hormones, with components of the luteal phase inducing changes similar to those observed in SerCa
specimens. Increased expression of SKIL, coupled with decreased expression of DAB2 in mutation carriers during this phase, could represent some of the earliest initiating or predisposing events of SerCa. Finally, specific loss of DAB2 in the secretory cells of the tubal epithelium during the luteal phase further highlights the relevance of this cell type in SerCa development.
CHAPTER 3

LOSS OF PROGESTERONE RECEPTOR ALPHA IN LUTEAL PHASE

FALLOPIAN TUBE EPITHELIUM

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This manuscript is currently in preparation for submission.

NOTES:

I completed all of the work presented in this chapter, with the exception of IHC which was performed by Kelvin So.
3.1: ABSTRACT

Purpose: To determine the expression of PR in SerCa as well as non-malignant FTE during the luteal and follicular phase, given our finding that FTEb collected during the luteal phase exhibit gene expression profiles most closely resembling that of SerCa specimens. This suggests that FTEb may respond differently to high circulating levels of progesterone during the luteal phase than FTEn and that this altered response may contribute to an increased propensity for malignant transformation.

Experimental Design: Expression of PR was examined in FTEn, FTEb and SerCa specimens using RT-qPCR and isoform-specific IHC on tissue arrays.

Results: RT-qPCR demonstrated decreased PR mRNA expression in SerCa and FTEb luteal samples compared to FTEn and FTEb follicular samples. Decreased nuclear expression of both PRA and PRB isoforms was observed in SerCa compared to FTEn. Nuclear PRA was also decreased in FTEn/b luteal samples overall relative to follicular samples. Expression of nuclear PRB was similar among all FTE groups, resulting in a decreased average PRA:B ratio during the luteal compared to follicular phase. Whereas PRA expression predominated in follicular FTE, PRB expression predominated in luteal samples. In addition, an increased proportion of luteal (45%) vs. follicular (14%) samples showed exclusive PRB cytoplasmic staining, potentially reflecting an enhanced activation of non-genomic signaling pathways involved in cell cycle progression. BRCA mutation status did not affect nuclear or cytoplasmic expression of PRA or PRB.

Conclusions: These results demonstrate differential expression of PR isoforms in FTE samples during the follicular and luteal phase that may underlie differences in gene expression profiles. However, similar relative isoform expression in FTEn and FTEb samples suggests that PR status is not a main contributor to predisposition to SerCa.
3.2: INTRODUCTION

High-grade SerCa is postulated to arise from the FTE. We have previously demonstrated that non-malignant FTE collected during the luteal phase from BRCA mutation carriers exhibit gene expression profiles more closely resembling that of ovarian and fallopian tube SerCa than FTE from control patients (229). This finding suggests that FTEb may respond differently to high circulating levels of progesterone during the luteal phase than FTEn and that this altered response may contribute to an increased propensity for malignant transformation.

Progesterone primarily signals through binding to intracellular PR, leading to receptor dimerization and binding to specific hormone response elements located in the promoter region of target genes to regulate transcription through interaction with co-regulatory proteins (113, 117, 119). Progesterone can also lead to rapid activation of a number of genes through protein-protein interactions involving cytoplasmic PR with components of the Src/MAPK, PI3K/Akt and JAK/Stat signaling pathways (114, 115, 119). The canonical PR is expressed as two isoforms, PRA and PRB, which are encoded by a single gene but arise by alternative use of two promoters (114). PRA and PRB are co-expressed at roughly equivalent levels in most target tissues (117). PRA is truncated at the amino terminal domain, and has been shown to exhibit differential functional properties compared to the full-length PRB isoform, including regulation of distinct genes (120) and differential effects on target tissues in knockout mice (113, 117).

Interestingly, PRA has been shown to act as a transdominant inhibitor of PRB (121, 123), and the relative expression of these two isoforms has been shown to vary in reproductive tissues as a result of development, hormonal status and carcinogenesis (113). Differential expression of PR isoforms in FTEb could thus underlie the altered gene expression profiles, particularly during the luteal phase when circulating progesterone levels are elevated.

The purpose of this study was to determine if PR isoform expression is altered in FTE from BRCA mutation carriers, particularly during the luteal phase. RT-qPCR and IHC using isoform-specific antibodies was therefore performed on patient samples of FTE and SerCa. To
our knowledge, this is the first comparative study of PR expression in non-malignant FTE throughout the ovarian cycle in BRCA1/2 mutation carriers and control patients.

3.3: MATERIALS AND METHODS

3.3.1. RT-qPCR for PR mRNA

RT-qPCR was performed (on the same samples used in Chapter 2) as described in Section 2.3.6 with the following modifications. Primer sequences used included PR forward 5'-GAACAGCGGATGAAAGAATCATC-3', PR reverse 5'-AGGAACTCTTCTTGGCTAACTTG-AAG-3', ACTB forward 5'-GCATTGTTACAGGAAGTCCCTTG-3' and ACTB reverse 5'-CTAT-CACCTCCCTGTGTGGA-3'. The PR primer pair used recognizes a specific sequence within the first 1,500bp from the poly(A) tail and would therefore amplify both PRA and PRB-specific mRNA in our samples (PR isoform-specific primers were not designed due to our use of 3'-biased linear amplification). Statistical analysis was done by one-way ANOVA followed by Tukey's Multiple Comparison test (p < 0.05). Individual samples were removed from the analysis if values were greater than three standard deviations from the mean.

3.3.2. PR isoform-specific IHC

IHC was performed using standard procedures as described in Section 2.3.7 with the following modifications. PRA protein was detected using mouse monoclonal anti-progesterone receptor antibody [PGR-312 (16), Novocastra Laboratories] (230) at a dilution of 1 in 200, while PRB protein was detected using anti-progesterone receptor (B-Form) antibody [PGR-B (SAN27), Novocastra Laboratories] at a dilution of 1 in 200. Scores for both nuclear PRA and PRB were given for percentage of epithelial cells stained (0, 0%; 1, 1-24%; 2, 25-75%; 3, >75%), as well as intensity of staining (0, negative; 1, light; 2, medium; 3, dark). The combined nuclear histoscore was obtained by adding these two individual scores as previously described. The presence or absence of cytoplasmic PRA or PRB positivity was noted for each case (not
quantified). Statistical analysis was done using one-way ANOVA followed by Tukey’s Multiple Comparison test (p < 0.05). Comparison of unpaired proportions was by Fisher’s exact test (p < 0.05).

3.4: RESULTS

3.4.1. Decreased expression of PR mRNA in FTEb and SerCa specimens

Analysis of previously obtained gene expression profiles of microdissected FTE and SerCa specimens (Chapter 2) revealed a similarly decreased expression of PR mRNA in FTEb luteal and SerCa samples relative to FTEb samples obtained during the follicular phase (p<0.001) (Figure 3.1A and Appendix II). A decrease in expression was also observed in FTEn luteal compared to follicular samples (p<0.05). Expression of PR mRNA was 2.2-fold lower in FTEb compared to FTEn samples within the luteal phase, although this did not reach statistical significance. No difference was observed in FTEb compared to FTEn samples within the follicular phase. Similar expression was also observed in FTEn samples overall and those FTEb previously found to cluster with FTEn (hereafter referred to as ‘FTEb(N)’), while a trend for decreased expression was observed in the four FTEb luteal specimens clustering with SerCa (‘FTEb (S)’, Figure 3.1B), suggesting that loss of PR mRNA may be an important and early event in serous carcinogenesis.

To confirm these findings, RT-qPCR was performed using the same select samples from each group used in Chapter 2. Similar to Affymetrix profiling, RT-qPCR revealed a trend towards decreased PR mRNA in luteal vs. follicular samples in both carriers and controls, as well as reduced expression in FTEb compared to FTEn samples within the luteal phase (Figure 3.1C). Unlike Affymetrix profiling, PR mRNA was also decreased in FTEb vs. FTEn samples within the follicular phase, suggesting that mutation of BRCA1/2 alone may partly determine PR mRNA levels. PR mRNA was barely detectable in SerCa specimens, with expression levels >4000-fold lower than that observed in FTEn follicular samples. Finally, FTEb samples overall
FIGURE 3.1: Decreased expression of PR mRNA in FTEb and SerCa specimens. Analysis of previously obtained (RMA-normalized) gene expression profiles revealed a decreased expression of PR in FTE luteal and SerCa samples compared to FTE follicular specimens (A). There was also a trend towards decreased expression in FTEb(S) relative to FTEb(N) samples (B). RT-qPCR of select samples revealed a trend towards decreased PR mRNA in SerCa and FTE in association with a BRCA mutation and/or during the luteal phase (C-D). Vertical bars in all panels represent average mRNA expression for each group of samples, with statistically significant differences in average mRNA expression indicated by different letters (one-way ANOVA with Tukey’s Multiple Comparison post-hoc test; p < 0.05). Circles in C and D indicate the individual sample values for RT-qPCR (relative to actin).
showed a trend towards decreased PR mRNA compared to FTEn samples, and expression in SerCa was >2000-fold lower than FTEn (p<0.05) (Figure 3.1D). In summary, PR mRNA was decreased in SerCa and non-malignant FTE in association with a BRCA mutation and/or during the luteal phase.

### 3.4.2. Increased expression of PRA- and PRB-influenced genes in FTEb luteal and FTEb(S) samples

In light of previous studies showing selective loss of either PRA or PRB isoforms in ovarian (124, 126) and breast (120, 123, 231, 232) cancers, my previously generated expression profiles were re-assessed using directed gene-specific analysis to determine the expression of PRA- vs. PRB-influenced genes. Altered expression of a large proportion of either subset of genes in FTEb luteal/FTEb(S) samples may suggest that these samples have preferentially lost one isoform. Examples of potential isoform-specific genes were taken from a previous study by Jacobsen et al (120), which describes gene expression profiling of ER+ PR-T47D breast cancer cell lines engineered to express PRA or PRB under the control of an inducible promoter. Control cells to which these profiles were compared were transfected with an empty construct. In this study, 46 distinct genes were found to be upregulated in PRB+ (relative to PR-null) T47D cells, while 32 were specifically upregulated in PRA+ cells.

Of the genes shown to be increased in PRB+ cells (120), 10/46 (21.7%) with corresponding probe sets on the Affymetrix GeneChip were unaltered in FTEb luteal vs. follicular samples (fold change <10%), whereas 22/46 (47.8%) and 14/46 (30.4%) showed increased or decreased expression in FTEb luteal respectively. In addition, 24/46 (52.2%) of PRB-upregulated genes (including 17 of the 22 increased in FTEb luteal samples) showed increased expression in FTEb(S) compared to FTEb(N) samples. When probe sets showing low levels of expression (all averages <50 RMA arbitrary units) were eliminated from the analysis, 7/30 (23.3%) were unaltered in FTEb luteal vs. follicular, while 14/30 (46.7%) and
9/30 (30%) showed increased or decreased expression in FTEb luteal samples. Of the filtered genes showing altered expression in FTEb luteal samples, 14/23 (60.9%) showed increased expression compared to FTEb follicular samples. Similarly, of filtered PRB-influenced genes with altered expression in FTEb(S) samples, 15/25 (60%) showed increased expression in FTEb(S) compared to FTEb(N) (including 13 genes also increased in FTEb luteal samples), suggesting that PRB signalling may be active in these cells. The specific PRB-upregulated genes showing increased expression in both the FTEb luteal and FTEb(S) subgroups are highlighted in Table 3.1.

Of the genes found to be increased in PRA+ cells by Jacobsen et al (120), 10/32 (31.3%) were unaltered in FTEb luteal compared to FTEb follicular samples, while 13/32 (40.6%) and 9/32 (28.1%) showed increased or decreased expression in FTEb luteal samples respectively. Furthermore, 15/32 (46.9%) of PRA-influenced genes (including all 13 increased in FTEb luteal samples) showed increased expression in FTEb(S) compared to FTEb(N) samples. When probe sets showing low levels of expression were eliminated, 5/17 (29.4%) were unaltered, 7/17 (41.2%) were increased and 5/17 (29.4%) were decreased in FTEb luteal compared to FTEb follicular samples. Of the filtered genes showing altered expression, 7/12 (58.3%) showed increased expression in FTEb luteal samples. These same 7 genes showed increased expression in FTEb(S) compared to FTEb(N) samples (7/11, 63.6% of altered genes in these samples). The specific PRA-upregulated genes showing increased expression in both the FTEb luteal and FTEb(S) subgroups are highlighted in Table 3.2. Altogether, gene expression profiles of FTEb luteal and FTEb(S) samples are consistent with active PRA and PRB signalling. It is therefore unclear through analysis of gene expression profiles alone whether these samples may have decreased expression of either or both PR protein isoforms.
Table 3.1. PRB-upregulated genes showing increased expression in FTEb luteal + FTEb(S) samples.

<table>
<thead>
<tr>
<th>Probe Set ID</th>
<th>Gene Title</th>
<th>Symbol</th>
<th>Fold Change FTEb luteal*</th>
<th>Fold Change FTEb(S)**</th>
</tr>
</thead>
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<tr>
<td>203973_s_at</td>
<td>GCAAT/enhancer binding protein (C/EBP), delta</td>
<td>CEBPD</td>
<td>2.80</td>
<td>3.55</td>
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<tr>
<td>203810_at</td>
<td>DnaJ (Hsp40) homolog, subfamily B, member 4</td>
<td>DNAJB4</td>
<td>1.13</td>
<td>1.13</td>
</tr>
<tr>
<td>204363_at</td>
<td>coagulation factor III</td>
<td>F3</td>
<td>1.34</td>
<td>1.84</td>
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<tr>
<td>213260_at</td>
<td>forkhead box C1</td>
<td>FOXC1</td>
<td>1.74</td>
<td>1.40</td>
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<tr>
<td>202724_s_at</td>
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<td>FOXO1A</td>
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<td>1.35</td>
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<tr>
<td>205662_at</td>
<td>gene regulated in breast cancer 1</td>
<td>GREB1</td>
<td>1.49</td>
<td>1.56</td>
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<tr>
<td>211538_s_at</td>
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<td>HSPA2</td>
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<td>1.26</td>
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<tr>
<td>203542_s_at</td>
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<td>KLF9</td>
<td>1.68</td>
<td>2.13</td>
</tr>
<tr>
<td>202726_at</td>
<td>ligase I, DNA, ATP-dependent</td>
<td>LIG1</td>
<td>1.91</td>
<td>1.24</td>
</tr>
<tr>
<td>205463_s_at</td>
<td>platelet-derived growth factor alpha polypeptide</td>
<td>PDGFA</td>
<td>1.80</td>
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<tr>
<td>202382_s_at</td>
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<td>PSID</td>
<td>1.38</td>
<td>1.45</td>
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<td>URB2</td>
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<td>1.56</td>
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<tr>
<td>210512_s_at</td>
<td>vascular endothelial growth factor</td>
<td>VEGF</td>
<td>1.89</td>
<td>2.20</td>
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* corresponds to the relative expression of the specific probe set in FTEb luteal samples compared to FTEb follicular.
** corresponds to the relative expression of the specific probe set in FTEb(S) samples compared to FTEb(N).
Table 3.2. PRA-upregulated genes showing increased expression in FTEb luteal + FTEb(S) samples.

<table>
<thead>
<tr>
<th>Probe Set ID</th>
<th>Gene Title</th>
<th>Symbol</th>
<th>Fold Change FTEb luteal*</th>
<th>Fold Change FTEb(S)**</th>
</tr>
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<td>39248_at</td>
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<td>AQP3</td>
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<td>2.10</td>
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<tr>
<td>201170_s_at</td>
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<td>BHLHB2</td>
<td>1.31</td>
<td>1.96</td>
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<td>GLS</td>
<td>1.56</td>
<td>1.21</td>
</tr>
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<td>methionine adenosyltransferase II, alpha</td>
<td>MAT2A</td>
<td>1.11</td>
<td>1.33</td>
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<tr>
<td>200632_s_at</td>
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<td>NDRG1</td>
<td>2.70</td>
<td>2.14</td>
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<td>209295_at</td>
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<td>TNFRSF10B</td>
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<td>1.48</td>
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<tr>
<td>218856_at</td>
<td>tumor necrosis factor receptor superfamily, member 21</td>
<td>TNFRSF21</td>
<td>2.85</td>
<td>4.19</td>
</tr>
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</table>

* corresponds to the relative expression of the specific probe set in FTEb luteal samples compared to FTEb follicular.
**corresponds to the relative expression of the specific probe set in FTEb(S) samples compared to FTEb(N).
3.4.3. Decreased PRA protein expression in luteal and SerCa specimens

IHC was performed using an antibody specifically recognizing PRA to determine the expression of this isoform in FTE and SerCa specimens. Contrary to what was expected based on the increased expression of a large proportion of PRA-influenced genes, there was a 1.4-fold decreased nuclear histoscore (% of cells staining + intensity) in luteal compared to follicular samples in both mutation carriers and controls (p<0.05, Figure 3.2A). A similar expression level was observed in FTEn vs. FTEb samples within the luteal or follicular phase, suggesting that PRA expression was influenced by the stage of the ovarian cycle rather than BRCA mutation status. Expression in SerCa was 4-fold lower than follicular samples, and 3-fold lower than luteal samples (p<0.001). No difference in PRA nuclear histoscore was observed in FTEn vs. FTEb samples overall (Figure 3.2B), while there was a trend for slightly decreased expression in FTEb(S) compared to FTEb(N) samples (Figure 3.2C).

In addition to nuclear staining, 6/12 (50%) of FTEn follicular and 10/16 (62.5%) of FTEb follicular samples showed presence of cytoplasmic PRA (Figure 3.2D). A small proportion of SerCa samples (2/52, 4%) also had PRA cytoplasmic staining, while no cytoplasmic localization was observed in FTEn/b luteal samples. In summary, nuclear PRA was decreased in SerCa and non-malignant FTE obtained during the luteal phase, and cytoplasmic staining was only observed in follicular samples. Representative staining for follicular, luteal and SerCa specimens is shown in Figure 3.2E-G.

3.4.4. Decreased PRB protein expression in SerCa specimens

IHC was also performed using an antibody specific to PRB. Unlike PRA, a similar average nuclear histoscore was observed in follicular compared to luteal samples for both BRCA mutation carriers and controls (Figure 3.3A). Nuclear PRB expression was also similar in FTEn compared to FTEb samples overall, while a 3.4-fold decrease was observed in SerCa specimens compared to FTEn (p<0.001, Figure 3.3B). In addition, there was no difference in
FIGURE 3.2: Decreased PRA protein expression in FTE luteal and SerCa specimens. IHC on tissue microarrays using an antibody that recognizes only PRA revealed a decreased nuclear histoscore in FTE luteal and SerCa relative to FTE follicular samples (A). In contrast, no change in average nuclear histoscore was observed in FTE\textsubscript{n} vs. FTE\textsubscript{b} samples overall (B), or among samples from previously profiled FTE\textsubscript{n} (denoted by *), FTE\textsubscript{b}(N) and FTE\textsubscript{b}(S) cases (C). In addition, while the majority of FTE follicular samples showed the presence of cytoplasmic PRA, this was not observed in FTE luteal and only rarely seen in SerCa samples (D). Representative PRA staining for follicular-phase FTE (E), luteal-phase FTE (F) and SerCa (G) samples are shown (magnification 20X). Statistically significant differences in average protein expression are indicated by different letters (one-way ANOVA with Tukey’s Multiple Comparison post-hoc test; p < 0.05).
**FIGURE 3.3: Decreased PRB protein expression in SerCa specimens.** IHC using a PRB-specific antibody revealed a decreased average nuclear histoscore in SerCa specimens relative to FTE, while no change was observed among FTE samples according to BRCA mutation status or stage of the ovarian cycle (A-B). Nuclear PRB expression levels were also similar in previously profiled FTEn (denoted by *), FTEb(N) and FTEb(S) cases (C). Cytoplasmic PRB staining was observed in a similar proportion of FTEn/b follicular and FTEn/b luteal samples, while only a small proportion of SerCa showed the presence of cytoplasmic PRB (D). Representative PRB staining for follicular FTE (E), luteal FTE (F) and SerCa (G) samples are shown (magnification 20X). Statistically significant differences in average protein expression are indicated by different letters (one-way ANOVA with Tukey’s Multiple Comparison post-hoc test; p < 0.05).
nuclear histoscore in FTEb(S) compared to FTEb(N) (Figure 3.3C). Cytoplasmic PRB was observed in 4/12 (33%) of FTEn follicular, 6/16 (37.5%) FTEb follicular and the same two SerCa samples showing cytoplasmic PRA positivity (Figure 3.3D). Unlike PRA, 5/11 (45%) of luteal samples from both carriers and controls showed the presence of cytoplasmic PRB. In summary, PRB nuclear and cytoplasmic expression was decreased in SerCa but not in non-malignant FTE samples. Representative staining for follicular, luteal and SerCa specimens is shown in Figure 3.3E-G.

3.4.5. Decreased nuclear PRA:B ratio in luteal relative to follicular samples

To determine the overall relative levels of nuclear PRA and PRB in FTE samples, the ratio of PRA to PRB nuclear histoscore (PRA:B) was calculated for each case. SerCa cases were excluded from the analysis, as a substantial proportion (30/52, 58%) had a PRA and/or PRB histoscore of 0. FTEb luteal specimens had an average PRA:B ratio 1.4-fold lower than FTEb follicular samples (p<0.05), with a similar trend also observed in normal controls (Figure 3.4A). No change in average PRA:B was observed in FTEn compared to FTEb samples overall (Figure 3.4B), or compared to FTEb(S) samples (Figure 3.4C). Consistent with an increased average PRA:B in follicular vs. luteal samples, the majority of individual follicular samples showed PRA predominance (indicated by a case ratio >1), while the opposite was observed in luteal samples (Figure 3.4D). A predominance of nuclear PRA was observed in 6/12 FTEn follicular and 11/16 FTEb follicular samples (61% overall), compared to 1/11 FTEn luteal and 2/11 FTEb luteal samples (14% overall) (p=0.001). In contrast, a predominance of nuclear PRB was seen in 6/11 FTEn luteal and 7/11 FTEb luteal samples (59% overall), compared to 1/12 FTEn follicular and 1/16 FTEb follicular samples (7% overall) (p=0.0001). In summary, FTE luteal samples had a decreased nuclear PRA:B ratio compared to FTE obtained during the follicular phase, while no difference was observed based on BRCA mutation status alone.
FIGURE 3.4: Decreased nuclear PRA:B in luteal samples. Comparison of PRA and PRB nuclear histoscores for each case revealed a decreased average PRA:B ratio in FTEb luteal compared to follicular samples, and a trend towards decreased PRA:B in FTEn luteal vs. follicular samples (A). There was no change in average PRA:B in FTEn vs. FTEb samples overall (B), or among previously profiled FTEn, FTEb(N) and FTEb(S) cases (C). While a large proportion (17/28, 61%) of follicular samples showed predominance of nuclear PRA (case ratio >1), PRB predominance (case ratio <1) was observed in a large proportion (13/22, 59%) of luteal samples (D).
3.4.6. Decreased PRA nuclear intensity in luteal samples

Consistent with the observed differences in overall nuclear histoscore, non-malignant FTE from the luteal and follicular phase showed differential intensity of PRA and PRB nuclear staining indicative of differential per-cell expression levels. All normal follicular samples (12/12) showed intense staining for PRA (indicated by a score of 3) compared to 0/11 normal luteal samples (p<0.0001, Figure 3.5A). Similar results were seen in mutation carriers, with a greater proportion of FTEb follicular (12/16, 75%) compared to FTEb luteal (1/11, 9%) samples showing intense nuclear PRA staining (p=0.001, Figure 3.5B). In contrast, similar proportions of FTEn follicular (5/12, 42%), FTEn luteal (4/11, 36%) and FTEb follicular (7/16, 44%) samples showed intense nuclear staining for PRB, while this was slightly decreased for FTEb luteal (2/11, 18%) samples (Figures 3.5C-D). Within follicular samples, a higher proportion of cases showed intense PRA (24/28, 86%) compared to intense PRB (12/28, 43%) staining (p=0.0018). On the other hand, within luteal samples, a slightly higher proportion of cases showed intense PRB (6/22, 27%) compared to intense PRA (1/22, 5%) staining, although this did not reach statistical significance.

3.4.7. Selective loss of cytoplasmic PRA in luteal samples

I next determined the overall relative levels of cytoplasmic PRA and PRB which could reflect a potential impact on activation of non-genomic signaling pathways. Approximately 50% of all groups of non-malignant FTE showed the presence of either both or neither PRA/B cytoplasmic staining (Figure 3.6). The majority of the remaining follicular samples from both carriers (4/12, 33%) and controls (6/16, 38%) showed the presence of only cytoplasmic PRA, while a small proportion showed only cytoplasmic PRB. In contrast, all of the remaining luteal samples (5/11 of both carriers and controls) showed the presence of only PRB cytoplasmic staining. No change in relative cytoplasmic staining was observed in FTEn vs. FTEb samples within the same stage of the ovarian cycle. Overall, an increased proportion of luteal (10/22,
FIGURE 3.5: Decreased PRA nuclear intensity in FTE luteal samples. The proportion of follicular and luteal cases with light (intensity score <3) and intense (score of 3) PRA (A-B) and PRB (C-D) nuclear staining is shown. While the majority of follicular samples from both control patients (A) and BRCA mutation carriers (B) showed intense PRA staining, this was only observed in one luteal sample (B). In contrast, intense PRB staining was observed in a similar proportion of FTEn follicular and luteal samples (C), and only a slightly decreased percentage of FTEb luteal vs. follicular samples were intensely positive for PRB (D).
FIGURE 3.6: Selective loss of cytoplasmic PRA in FTE luteal samples. Approximately half of FTE samples from all groups (irrespective of mutation status or stage of the ovarian cycle) did not show predominant expression of either cytoplasmic PRA or PRB (indicated by the presence of neither or both isoforms). Of the remaining samples, a large proportion of follicular samples showed only cytoplasmic PRA staining while all of the luteal samples showed only cytoplasmic PRB.
45%) compared to follicular (4/28, 14%) samples showed exclusive PRB cytoplasmic staining (p<0.05). Altogether, luteal samples showed predominance of cytoplasmic in addition to nuclear PRB staining.

3.5: DISCUSSION

To determine if differential expression of PR in FTEb could contribute to the altered gene expression profiles observed in BRCA mutation carriers during the luteal phase, analysis of PR mRNA and protein expression was performed. Affymetrix profiling and RT-qPCR revealed significantly decreased PR mRNA in SerCa and FTEb luteal relative to FTEb follicular samples. There was also a trend towards decreased expression in FTEb(S) and FTEb overall compared to normal control FTE. Finally, RT-qPCR showed a trend towards decreased PR mRNA in FTEb vs. FTEn samples within both the follicular and luteal phase, suggesting that BRCA mutation status in addition to the luteal phase milieu affects PR mRNA levels. Several studies have previously reported decreased expression of total PR in ovarian and fallopian tube cancer. Lau et al demonstrated a marked reduction of PR mRNA in ovarian cancer cell lines compared to primary cultures of normal OSE cells using semi-quantitative RT-PCR (233), while others have found low PR protein expression in both BRCA1-associated and sporadic serous ovarian cancers by IHC (30). Expression of PR has been found to be a favourable prognostic marker in ovarian cancer (234), but may simply reflect the increased expression in endometrioid (64% of cases considered PR+) compared to serous (25% PR+) tumours in the study, as endometrioid tumours are characterized by greatly enhanced survival (12). Finally, one study observed slightly decreased PR protein expression in fallopian tube carcinomas compared to areas of benign FTE in patients with or without a BRCA mutation (27).

Our finding of decreased total PR mRNA in non-malignant FTE in association with the presence of a BRCA mutation is the first reported. Previous studies have focused on determining total PR protein expression in normal FTE and OSE from mutation carriers and
controls, with no differential expression noted (187, 191). PR protein was expressed abundantly in both ciliated and secretory FTE cells, and expression in morphologically normal and dysplastic areas of mutation carriers was similar (191). In studies of BRCA-associated ovarian epithelium, the only differences noted were an increased expression of PR protein in inclusion cyst epithelium compared to OSE, which the authors attributed to an increased exposure to ovarian stroma-derived hormones including estrogen (187). Interestingly, PR expression was higher in inclusion cyst epithelium from mutation carriers compared to controls, potentially consistent with the regulation of ER transactivation activity by wild-type BRCA1 (51, 52) and in contrast to the low expression observed in ovarian SerCa in this and other studies.

The finding that PR mRNA is lowest during the luteal phase (in both carriers and controls) is consistent with previous studies of normal human FTE throughout the ovarian cycle, which have shown little or no expression during this phase using semi-quantitative RT-PCR (235). Similar results have also been obtained for total PR protein, and the sharp decline in (cytosolic) PR was found to coincide with the luteal rise of serum progesterone concentration in each study (236-238). This is consistent with studies demonstrating the polyubiquitination and subsequent degradation of PR protein upon exposure to progesterone (239). Thus the decline in PR expression observed in this study likely represents downregulation of PR.

Previous studies have demonstrated that the relative levels of PRA and PRB influence the response of target cells to progesterone. For instance, breast cancer cells with predominant expression of PRA have shown an exaggerated proliferative response to luteal-phase levels of progesterone compared to cells with predominant expression of PRB (240). Isoform-specific IHC was therefore performed to determine if the decreased overall levels of PR in FTEb luteal/FTEb(S) samples represented a preferential loss of the PRA or PRB isoform. Selective loss of either isoform in FTEb would potentially lead to an altered response to progesterone during the luteal phase which may contribute to SerCa development. Contrary to what we expected based on increased expression of many PRA-influenced genes in FTEb luteal and FTEb(S) samples, IHC analysis revealed a decreased nuclear intensity and overall histoscore
of nuclear PRA protein in non-malignant FTE during the luteal phase in both mutation carriers and normal controls, as well as a trend towards decreased histoscore in FTEb(S). Conversely, no change in average expression of nuclear PRB protein was observed in these sample groups, resulting in a decreased average PRA:B ratio in luteal vs. follicular samples. In addition, the majority of individual luteal samples showed PRB predominance, while PRA was predominant in the majority of follicular samples. This is consistent with a previous study which found a higher expression of total PR protein (PRA+B) compared to PRB during the follicular phase in human normal FTE (235).

In contrast to two earlier studies in ovarian cancer, SerCa samples showed similarly reduced levels of both PRA and PRB protein relative to control (FTEn) samples. As previously discussed, Akahira et al demonstrated a lower expression of PRA compared to PRB for all EOC histotypes using both RT-PCR and IHC; relative isoform levels were also unchanged according to grade or stage of tumour (126). The same group found sequential specific down-regulation of PRA, but not PRB, from normal ovarian epithelium through benign, borderline and malignant serous ovarian tumours, although the grade of the malignant tumours was not indicated (124). In normal OSE and serous adenomas both isoforms were equally expressed, whereas PRB predominated in borderline and malignant tumours. Similar studies should be performed in the fallopian tube, to determine relative isoform levels in potential SerCa precursors such as p53 signatures and TICs. Finally, in a study comparing sporadic and BRCA1-associated ovarian SerCa, nuclear PRA was expressed in only 2/22 (9%) of hereditary in comparison to 8/22 (36%) of sporadic cases while nuclear PRB was similarly expressed in both groups (30), emphasizing the potential role of relative PR isoform expression in BRCA1-associated tumourigenesis.

Interestingly, while the decreased relative expression of PRA to PRB nuclear protein in luteal-phase FTE is consistent with that previously observed in ovarian cancer, the opposite trend has been observed in breast cancer. While PRA and PRB are expressed at roughly equivalent levels in normal breast epithelium (similar to FTEn overall in this study), several
studies have found a predominance of PRA in a high proportion of invasive breast tumours (123, 231, 232) as well as ductal carcinomas in situ (123). A specific lack of expression of the PRB isoform has also been observed in normal breast epithelium obtained from BRCA1/2 mutation carriers compared to control patients, resulting in predominate expression of PRA in 40% of cases (125). It is noteworthy, however, that these breast samples had fewer epithelial cells positive for PRA or PRB compared to FTE overall. Whereas breast samples obtained from mutation carriers or controls showed a median percentage of positive cells of 3-30% (125), 36/58 (62%) and 31/58 (53%) of all FTE samples in our study showed >75% positivity for nuclear PRA or PRB respectively. In addition, whereas relative levels of PRA and PRB in the FTE were altered in the luteal vs. follicular phase, equimolar expression was maintained throughout the cycle in normal breast epithelium (123). These key differences suggest that the expression, regulation and potentially function of these isoforms is distinct in these tissues, similar to previous reports comparing the uterus and breast (113). It is therefore possible that, unlike breast epithelial cells, FTE cells with predominant expression of PRB may show an exaggerated proliferative response to luteal progesterone.

Interestingly, despite a significant loss in PRA protein expression, FTEb luteal samples showed an increased expression of genes previously shown to be upregulated in cells expressing only PRA (120). This discrepancy can potentially be explained in three ways. Firstly, the differential expression of these genes has not been validated using an additional approach such as RT-qPCR and thus the possibility that this is a false positive result cannot be excluded. Secondly, these genes were found to be induced in a cell line expressing only PRA, while reports have suggested that the progesterone response may be more complex in cells expressing both isoforms. One study found that transcriptional activities of PR heterodimers and homodimers are distinct, and further that the relative levels of PRA and PRB is as important at determining the response to progesterone as total PR levels (118). In fact, when T47D breast cancer cells expressing similar levels of PRA and PRB were made to over-express PRA, expression of TNFRSF10B was reduced. Thus, the presence of PRB alters the
impact of PRA on gene expression. Thirdly, a number of the genes influenced by progesterone through PRA (e.g. AQP3, BHLHB2, TNFRSF10B) have also been shown to be influenced by other hormones increased during the luteal phase, most notably glucocorticoids (241-243).

In addition to changes in nuclear PR isoforms, luteal-phase FTE showed altered relative levels of cytoplasmic PRA and PRB compared to follicular-phase FTE. While cytoplasmic PRB staining was similarly observed in all FTE, cytoplasmic PRA was only present in follicular samples. Overall, an increased proportion of luteal compared to follicular samples showed exclusive PRB cytoplasmic staining, with similar findings in both mutation carriers and controls. This suggests that luteal phase samples may have enhanced activation of non-genomic signaling pathways thought to be primarily mediated by PRB. Following progesterone treatment of breast cancer cells, endogenous extra-nuclear PRB rapidly activates Src/MAPK pathways through protein-protein interactions, leading to cell cycle progression (114, 244). Interestingly, preliminary experiments have also demonstrated a proliferative effect of progesterone on primary cultures derived from human FTE, although the mechanisms involved (or the impact of relative PR isoform expression) have not been investigated (Tone, unpublished). As the inhibitory function of PRA does not require DNA binding (121), it is possible that the non-genomic aspect of PRB signaling is inhibited by cytoplasmic PRA in follicular but not luteal samples. However, the ability of PRA to inhibit PRB has been found to be model-, cell- and promoter-specific (121, 122), and forced cytoplasmic expression of PRA in breast cancer cells led to Src activation similar to cytoplasmic PRB (114).

In summary, expression of PR mRNA was found to be decreased in SerCa and BRCA-mutated FTE, especially during the luteal phase. SerCa specimens also exhibited decreased expression of nuclear PRA and PRB protein compared to normal controls. FTE luteal samples obtained from both BRCA mutation carriers and controls showed a preferential loss of nuclear and cytoplasmic PRA in comparison to FTE obtained during the follicular phase. The resulting PRB predominance in luteal samples may contribute to the differential gene expression profiles observed during this phase. However, similar relative isoform expression in FTEa and FTEb
(as well as in FTEb(N) and FTEb(S)) samples suggests that differential response to progesterone is not a likely determinant of predisposition to SerCa, and that an additional component of the luteal phase milieu may be involved.
CHAPTER 4

ALTERED GR AND NFκB INFLAMMATORY SIGNALLING IN POST-OVULATORY FTEB(S) AND SERCA SAMPLES

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This manuscript is currently in preparation for submission.

NOTES:

I completed all of the work presented in this chapter, with the exception of IHC which was performed by Kelvin So and clustering which was performed by Carl Virtanen.
4.1: ABSTRACT

**Purpose:** To characterize the expression of GR, NFκB and their respective targets in SerCa and non-malignant FTE during the luteal and follicular phase of the ovarian cycle to determine if altered inflammatory signalling during the luteal phase may contribute to an increased propensity for malignant transformation.

**Experimental Design:** Expression profiles generated in chapter 2 were re-assessed using directed gene-specific analysis to determine the mRNA expression of GR, the active NFκB subunit RelA and their respective targets in FTEn, FTEb and SerCa specimens. Expression of GR and RelA protein was determined by IHC on tissue microarrays.

**Results:** Statistical analysis of gene expression profiles indicated an elevated expression of GR mRNA in FTEb(S) relative to FTEb(N) samples. FTEb(S) samples also showed an increased percentage of positive cells for nuclear GR protein compared to FTEb(N), possibly reflective of diminished GR signalling (which typically leads to GR downregulation). Consistently, FTEb(S) samples showed altered expression of 20/55 (36%) and 43/62 (69%) of genes previously found to be directly upregulated and downregulated by GR respectively. FTEb(S) samples also showed an increased expression of RelA mRNA, and percentage of positive cells for both nuclear and cytoplasmic RelA protein. Intense RelA staining was specifically observed in secretory FTE cells, the proposed cell-type of origin for SerCa which was previously found to show a specific loss of DAB2 during the luteal phase. Cluster analysis using a cassette of inflammatory mediators positively regulated by NFκB separated FTEb(S) and SerCa from FTEb(N) samples, implicating altered inflammatory signalling during the luteal phase as a risk factor for malignant transformation.

**Conclusions:** Gene expression profiles of post-ovulatory FTEb(S) and SerCa samples are consistent with elevated pro-inflammatory signalling and disrupted GR signalling.
4.2: INTRODUCTION

In addition to increased progesterone secretion, the luteal phase is characterized by an elevation of ovulation-associated inflammatory cytokines such as TNFα and IL-1 at the ovarian surface and presumably the adjacent FTE (91). Binding of these cytokines to their cell surface receptors leads to the release of cytoplasmic NFκB subunits from inhibitory proteins, translocation to the nucleus and activation of gene transcription by NFκB dimers (145, 147, 151, 152). Genes induced by NFκB include cytokines and their respective receptors, chemokines responsible for recruitment of inflammatory leukocytes, cell adhesion molecules and genes involved in regulation of cell proliferation and prevention of apoptosis (100, 147, 151). Chronic activation of the inflammatory response by NFκB can increase the risk for tumour development via oxidative stress and DNA damage (95, 102, 154, 155), consistent with the protective role of anti-inflammatory drugs against ovarian cancer development (91, 99).

In addition to activation of NFκB, ovulation-associated cytokines induce a compensatory anti-inflammatory response through enhanced cortisol production in the periovulatory follicle and on the ovarian surface. This is achieved by cytokine-induced upregulation of 11βHSD type I, the enzyme responsible for converting the inactive precursor cortisone to active cortisol (91, 99). Cortisol passes through the plasma membrane leading to the formation of cytoplasmic ligand-GR complexes, dissociation from co-chaperone proteins and translocation of GR homodimers into the nucleus resulting in regulation of pro- and anti-inflammatory target gene transcription. In addition, cytoplasmic ligand-GR monomers can directly interact with the active NFκB subunit RelA, leading to suppressed synthesis of inflammatory mediators (148, 149, 245, 246). Thus, activation of GR signalling by ovulation-associated cytokines ultimately leads to resolution of the inflammatory process, which may protect cells from tumour development.

I hypothesize that GR anti-inflammatory signalling is disrupted during SerCa development. The purpose of this study was therefore to characterize the expression of GR, NFκB and their respective targets in SerCa and non-malignant FTE during the luteal and
follicular phase of the ovarian cycle to determine if altered inflammatory signalling during the luteal phase may contribute to an increased propensity for malignant transformation.

4.3: MATERIALS AND METHODS

4.3.1. IHC for GR and RelA

IHC was performed as described in Section 2.3.7 with the following modifications. GR was detected using mouse monoclonal anti-glucocorticoid receptor antibody [GR (3D5), Santa Cruz Biotechnology] at a dilution of 1 in 50. Scores for nuclear GR were given for percentage of epithelial cells stained (0, <10%; 1, 10-14%; 2, 15-19%; 3, ≥20%), as well as intensity of staining (0, negative; 1, light; 2, medium; 3, dark). RelA was detected using rabbit polyclonal anti-p65 antibody [NFκB p65 (A), Santa Cruz Biotechnology] at a dilution of 1 in 300. Scores were given for percentage (0, ≤10%; 1, 11-15%; 2, 16-20%; 3, >20%) and intensity (0, negative; 1, light; 2, medium; 3, dark) of nuclear RelA staining. Separate scores were also given for percentage (0, <70%; 1, 70-79%; 2, 80-89%; 3, ≥90%) and intensity (0, negative; 1, light; 2, medium; 3, dark) of cytoplasmic RelA. Combined histoscores for nuclear GR, nuclear RelA and cytoplasmic RelA were each calculated by adding the corresponding percentage and intensity scores as previously described. Statistical analysis was done using one-way ANOVA followed by Newman-Keuls Multiple Comparison test (p < 0.05). Comparison of unpaired proportions was done using Fisher’s Exact test (p < 0.05).

4.3.2. Hierarchical clustering of samples by cassette of NFκB target genes

A list of potential NFκB target genes was compiled in collaboration with bioinformatics specialist Carl Virtanen through extensive literature review (146, 247-250), and the corresponding probe sets on the Affymetrix GeneChip U133A Plus 2.0 were identified. Following initial identification of potential targets by review articles, primary papers for each gene were examined to further confirm its regulation by NFκB and determine whether NFκB
positively or negatively regulated its expression. Although the majority of genes were independently identified as NFκB targets in multiple studies, genes identified in only one study were included for the purposes of this analysis. The final list of 258 probe sets (corresponding to 144 genes positively influenced by NFκB) and all CEL files were imported into GeneSpringGX (version 10.0, Agilent). Normalization was performed, first by RMA and then by the median measurement for each probe set across all samples. Two-way hierarchical cluster analysis with average linkage using a Pearson centered similarity distance metric was then performed. Cluster results were then exported to Excel for further viewing and annotation.

4.4: RESULTS

4.4.1. Increased expression of GR mRNA and nuclear protein in FTEb(S)

Analysis of previously obtained gene expression profiles (Chapter 2) revealed a 1.5-fold elevated mRNA expression of the gene encoding for GR, nuclear receptor subfamily 3, group C, member 1 (NR3C1) in FTEb luteal samples relative to FTEb samples obtained during the follicular phase (p<0.01) (Figure 4.1A). Similar expression was observed in FTEb follicular and normal control samples from both the luteal and follicular phase, suggesting that a combination of the luteal phase milieu with BRCA mutation status contributed to elevated GR levels. A 1.6-fold increased expression was also observed in FTEb(S) compared to FTEb(N) and FTEn samples (p<0.05) (Figure 4.1B and Appendix I). Expression in FTEb(S) samples was similar to SerCa, while a 1.4-fold increase was observed relative to all other luteal samples presumably exposed to similar levels of post-ovulatory cortisol (p=0.0069) (Figure 4.1C). This elevated GR mRNA expression in FTEb(S) samples led us to postulate that these samples have disrupted GR signalling, given previous reports that functional GR signalling leads to GR downregulation in several cell types (145, 160, 251, 252).

IHC was then performed on tissue microarrays to determine the relative level and subcellular localization of GR protein in FTEn, FTEb(N), FTEb(S) and SerCa samples. While
FIGURE 4.1: Increased expression of GR mRNA in FTEb(S) samples. Analysis of my previously generated gene expression profiles revealed an increased expression of GR in FTEb luteal relative to FTEb follicular samples (A). GR was also found to be elevated in FTEb(S) samples compared to FTEn and FTEb(N) (B), as well as compared to all other luteal samples (C). Expression in SerCa was similar to that observed in FTEb(S) samples (B). Statistically significant differences in average mRNA expression are indicated by different letters (as determined by one-way ANOVA followed by Newman-Keuls Multiple Comparison post-hoc test; p < 0.05).
negligible cytoplasmic staining was present in all groups, nuclear positivity was observed in both secretory and ciliated FTE and carcinoma cells. FTEb(S) samples had a slightly increased average nuclear histoscore (3.75±0.63) compared to FTEn (3.08±0.42) and FTEb(N) (2.88±0.13) samples, although this did not reach statistical significance (Figure 4.2A). Consistently, 3/4 (75%) of FTEb(S) samples had a percentage score ≥2 (corresponding to ≥15% positivity) compared to 4/12 (33%) of FTEn and 0/8 of FTEb(N) samples (p=0.0182) (Figure 4.2B). Interestingly, despite similar levels of GR mRNA in SerCa and FTEb(S) samples, SerCa samples had a lower average nuclear histoscore (0.76±0.18) compared to the non-malignant FTE groups (p<0.001) and only 5/51 (10%) had a percentage score ≥2 (Figures 4.2A-B). Representative staining for FTEb(N), FTEb(S) and SerCa samples are shown in Figures 4.2C-E. Overall, FTEb(S) samples showed an increased expression of GR mRNA and percentage of positive cells for nuclear GR protein compared to FTEb(N), potentially reflective of diminished GR signalling in these samples.

4.4.2. Altered expression of GR-influenced genes in FTEb(S) samples

To further explore the hypothesis that GR signalling was specifically disrupted in FTEb(S) samples, expression profiles were re-assessed using directed gene-specific analysis to determine the expression of GR-influenced genes. Review of the literature (including both review and primary research papers) revealed 80 genes previously shown to be induced by glucocorticoids (145, 158, 159, 242, 251, 253-256). Similar to the compilation of potential NFκB targets described in Section 4.3.2, identification of genes in one study was sufficient for inclusion as a potential GR target gene for the purposes of this analysis. Prior to filtering for level of expression of the corresponding probe set, 10/80 (12.5%) of these genes were unaltered in FTEb(S) vs. FTEb(N) samples (fold change <10%), while 43/80 (53.8%) and 27/80 (33.8%) showed increased or decreased expression in FTEb(S) respectively. Of the genes showing altered expression in FTEb(S), 43/70 (61.4%) show increased expression while 27/70 (38.6%) show decreased expression compared to FTEb(N).
FIGURE 4.2: Increased expression of nuclear GR protein in FTEb(S) samples. IHC for GR on tissue microarrays revealed a trend towards increased average nuclear histoscore in samples obtained from previously profiled FTEb(S) relative to FTEb(N) and FTEn cases (A), whereas expression in SerCa samples was lower than in all non-malignant FTE groups. When percentage of epithelial cells stained was considered separately, a greater proportion of FTEb(S) samples were given a score ≥2 compared to FTEb(N), FTEn and SerCa samples (B). Representative GR staining for FTEb(N) (C), FTEb(S) (D) and SerCa (E) samples is shown (magnification 20X). Statistically significant differences in average GR protein expression in A are indicated by different letters (one-way ANOVA followed by Newman-Keuls Multiple Comparison post-hoc test; p < 0.05).
When probe sets showing low levels of expression similar to background (all averages <50 RMA arbitrary units) were eliminated, 5/60 (8.3%) were unaltered, while 35/60 (58.3%) and 20/60 (33.3%) showed increased or decreased expression in FTEb(S) samples. Of the filtered genes showing altered expression in FTEb(S), 35/55 (63.6%) showed increased expression while 20/55 (36.4%) showed decreased expression compared to FTEb(N). Functional categories of altered genes are outlined in Table 4.1. While genes involved in cell growth and apoptosis, inflammation and/or the immune response, metabolism and transport were largely increased as expected, a number of glucocorticoid-induced genes involved in regulation of transcription (2/3, 66.7%) and signal transduction (6/11, 54.5%) showed decreased expression in FTEb(S). Specific genes typically upregulated by glucocorticoids showing decreased expression in FTEb(S) are highlighted in Table 4.2. Notably, 13/20 (65%) of these probe sets also showed decreased expression in SerCa compared to FTEb(N) samples.

Review of the literature also revealed 121 genes previously shown to be decreased by glucocorticoids (159, 163, 242, 251, 253-255, 257). Prior to filtering for level of expression of the corresponding probe set, 31/121 (25.6%) of these genes were unaltered in FTEb(S) vs. FTEb(N) samples, while 65/121 (53.7%) and 25/121 (20.7%) showed increased or decreased expression in FTEb(S) respectively. Of the genes showing altered expression in FTEb(S), 65/90 (72.2%) showed increased expression while 25/90 (27.8%) showed decreased expression compared to FTEb(N).

When probe sets showing low levels of expression were eliminated, 14/76 (18.4%) were unaltered, while 43/76 (56.6%) were increased and 19/76 (25%) were decreased in FTEb(S) samples. Finally, of the filtered genes showing altered expression in FTEb(S), 43/62 (69.4%) showed increased expression while 19/62 (30.6%) showed decreased expression compared to FTEb(N). Functional categories of altered genes are outlined in Table 4.3. Interestingly, ≥50% of GR-repressed genes within all functional categories showed increased expression in FTEb(S) relative to FTEb(N) samples. Genes involved in inflammation and/or immune
Table 4.1. Functional categories of altered GR-induced genes.

<table>
<thead>
<tr>
<th>Category</th>
<th>Total No.</th>
<th>NA*</th>
<th>NC**</th>
<th>No. of changers</th>
<th>No. (%) increased*</th>
<th>No. (%) decreasedd</th>
</tr>
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<tbody>
<tr>
<td>Cell growth &amp; apoptosis</td>
<td>14</td>
<td>3</td>
<td>1</td>
<td>10</td>
<td>8 (80)</td>
<td>2 (20)</td>
</tr>
<tr>
<td>Inflammation/immune response</td>
<td>15</td>
<td>3</td>
<td>1</td>
<td>11</td>
<td>8 (72.7)</td>
<td>3 (27.3)</td>
</tr>
<tr>
<td>Metabolism</td>
<td>5</td>
<td>2</td>
<td>0</td>
<td>3</td>
<td>2 (66.7)</td>
<td>1 (33.3)</td>
</tr>
<tr>
<td>Regulation of transcription</td>
<td>5</td>
<td>2</td>
<td>0</td>
<td>3</td>
<td>1 (33.3)</td>
<td>2 (66.7)</td>
</tr>
<tr>
<td>Signal transduction</td>
<td>18</td>
<td>4</td>
<td>3</td>
<td>11</td>
<td>5 (45.5)</td>
<td>6 (54.5)</td>
</tr>
<tr>
<td>Transport</td>
<td>8</td>
<td>1</td>
<td>0</td>
<td>5</td>
<td>4 (80)</td>
<td>1 (20)</td>
</tr>
<tr>
<td>Other/unknown</td>
<td>17</td>
<td>5</td>
<td>0</td>
<td>12</td>
<td>7 (58.3)</td>
<td>5 (41.7)</td>
</tr>
</tbody>
</table>

*corresponds to number of genes with low levels of expression similar to background. **corresponds to genes with unchanged expression in FTEb(S) vs. FTEb(N). *corresponds to genes increased in FTEb(S), while *indicates genes decreased in FTEb(S) relative to FTEb(N) samples.
Table 4.2. Genes upregulated by GR showing decreased expression in FTEb(S) samples.

<table>
<thead>
<tr>
<th>Probe Set ID</th>
<th>Gene Title</th>
<th>Symbol</th>
<th>Fold Change*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell Growth &amp; Apoptosis (2)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>209028_s_at</td>
<td>abl-interactor 1</td>
<td>ABI1</td>
<td>0.89</td>
</tr>
<tr>
<td>213139_at</td>
<td>snail homolog 2</td>
<td>SNAI2</td>
<td>0.80</td>
</tr>
<tr>
<td><strong>Inflammation/Immune Response (3)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>205476_at</td>
<td>chemokine (C-C motif) ligand 20</td>
<td>CCL20</td>
<td>0.80</td>
</tr>
<tr>
<td>204767_s_at</td>
<td>flap structure-specific endonuclease 1</td>
<td>FEN1</td>
<td>0.83</td>
</tr>
<tr>
<td>204731_at</td>
<td>transforming growth factor, beta receptor III</td>
<td>TGFBR3</td>
<td>0.47</td>
</tr>
<tr>
<td><strong>Metabolism (1)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>223652_at</td>
<td>arsenic (+3 oxidation state) methyltransferase</td>
<td>AS3MT</td>
<td>0.87</td>
</tr>
<tr>
<td><strong>Regulation of Transcription (2)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>214532_x_at</td>
<td>POU class 5 homeobox 1</td>
<td>POU5F1</td>
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<td>207801_s_at</td>
<td>ring finger protein 10</td>
<td>RNF10</td>
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<tr>
<td><strong>Signal Transduction (6)</strong></td>
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</tr>
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<td>208325_s_at</td>
<td>A kinase (PRKA) anchor protein 13</td>
<td>AKAP13</td>
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<tr>
<td>209296_at</td>
<td>CDC42 effector protein (Rho GTPase binding) 3</td>
<td>CDC42EP3</td>
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<td>224828_at</td>
<td>cytoplasmic polyadenylation element binding protein 4</td>
<td>CPEB4</td>
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<td>220161_s_at</td>
<td>erythrocyte membrane protein band 4.1 like 4B</td>
<td>EPB41L4B</td>
<td>0.33</td>
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<td>226907_at</td>
<td>protein phosphatase 1, regulatory (inhibitor) subunit 14C</td>
<td>PP1R14C</td>
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<tr>
<td>202084_s_at</td>
<td>SEC14-like 1</td>
<td>SEC14L1</td>
<td>0.84</td>
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<td><strong>Transport (1)</strong></td>
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<td></td>
<td></td>
</tr>
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<td>SCNN1A</td>
<td>0.70</td>
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<td><strong>Other/Unknown (5)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>221815_at</td>
<td>abhydrolase domain containing 2</td>
<td>ABHD2</td>
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<tr>
<td>214911_s_at</td>
<td>bromodomain containing 2</td>
<td>BRD2</td>
<td>0.76</td>
</tr>
<tr>
<td>216435_at</td>
<td>DnaJ (Hsp40) homolog, subfamily C, member 15</td>
<td>DNAJC15</td>
<td>0.75</td>
</tr>
<tr>
<td>207291_at</td>
<td>proline rich Gla (G-carboxyglutamic acid) 4 (transmembrane)</td>
<td>PRRG4</td>
<td>0.74</td>
</tr>
<tr>
<td>208805_at</td>
<td>proteasome (prosome, macropain) subunit, alpha type, 6</td>
<td>PSMA6</td>
<td>0.81</td>
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</tbody>
</table>

* corresponds to the relative expression of the specific probe set in FTEb(S) samples compared to FTEb(N) (decreased expression <0, increased expression >0). Italicized probe sets also show decreased expression in SerCa relative to FTEb(N) samples.
Table 4.3. Functional categories of altered GR-repressed genes.

<table>
<thead>
<tr>
<th>Category</th>
<th>Total No.</th>
<th>NA*</th>
<th>NC**</th>
<th>No. of changers</th>
<th>No. (%) increased*</th>
<th>No. (%) decreasedd</th>
</tr>
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<tbody>
<tr>
<td>Cell growth &amp; apoptosis</td>
<td>19</td>
<td>5</td>
<td>5</td>
<td>9</td>
<td>7 (77.8)</td>
<td>2 (22.2)</td>
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<tr>
<td>Inflammation/immune response</td>
<td>47</td>
<td>27</td>
<td>0</td>
<td>20</td>
<td>16 (80)</td>
<td>4 (20)</td>
</tr>
<tr>
<td>Metabolism</td>
<td>7</td>
<td>2</td>
<td>1</td>
<td>4</td>
<td>2 (50)</td>
<td>2 (50)</td>
</tr>
<tr>
<td>Regulation of transcription</td>
<td>20</td>
<td>5</td>
<td>3</td>
<td>12</td>
<td>8 (66.7)</td>
<td>4 (33.3)</td>
</tr>
<tr>
<td>Signal transduction</td>
<td>8</td>
<td>3</td>
<td>2</td>
<td>3</td>
<td>2 (86.7)</td>
<td>1 (33.3)</td>
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<tr>
<td>Transport</td>
<td>5</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>2 (86.7)</td>
<td>1 (33.3)</td>
</tr>
<tr>
<td>Other/unknown</td>
<td>15</td>
<td>2</td>
<td>2</td>
<td>11</td>
<td>6 (54.5)</td>
<td>5 (45.5)</td>
</tr>
</tbody>
</table>

*corresponds to number of genes with low levels of expression similar to background. **corresponds to genes with unchanged expression in FTEb(S) vs. FTEb(N). *corresponds to genes increased in FTEb(S), while *indicates genes decreased in FTEb(S) relative to FTEb(N) samples.
response were especially altered, with 16/20 (80%) of these genes showing increased expression. Specific genes typically downregulated by glucocorticoids showing increased expression in FTEb(S) are highlighted in Table 4.4. Notably, 35/43 (81.4%) of these genes are also increased in SerCa relative to FTEb(N). Overall, FTEb(S) samples showed altered expression of a subset of GR-induced genes and a large proportion of GR-repressed genes, most notably genes involved in negative regulation of inflammatory signalling by GR.

4.4.3. Increased expression of RelA mRNA and protein in FTEb(S) samples

Similar to GR, previous analysis of gene expression profiles revealed a 1.9-fold increased mRNA expression of the active NFκB subunit RelA in FTEb luteal relative to FTEb follicular samples (p<0.001)(Figure 4.3A and Appendix II). RelA expression was similar in FTEn vs. FTEb follicular samples, while FTEn luteal samples showed 1.4-fold lower levels compared to FTEb luteal samples (p<0.01), suggesting that a combination of the luteal phase milieu and BRCA mutation status also affect RelA levels. RelA mRNA expression was also 1.9-fold higher in luteal FTEb(S) samples compared to FTEn and FTEb(N) samples (p<0.001)(Figure 4.3B and Appendix I). Unlike GR mRNA, RelA was not similarly elevated in SerCa specimens. Finally, FTEb(S) samples showed a 1.7-fold increased RelA expression relative to all other luteal samples (p=0.0008) (Figure 4.3C).

IHC was then performed on tissue microarrays to determine the relative level and subcellular localization of RelA protein in FTEn, FTEb(N), FTEb(S) and SerCa samples. IHC revealed a trend towards FTEb(S) samples showing an increased average nuclear histoscore (5.00±0.41) compared to FTEn (4.25±0.48) and FTEb(N) (4.25±0.53) samples (Figure 4.4A). Consistently, a slightly increased proportion of FTEb(S) (4/4, 100%) had a nuclear percentage score ≥2 (corresponding to >15% positivity) compared to FTEn (8/12, 66.7%) and FTEb(N) (5/8, 62.5%) samples, although this did not reach statistical significance (Figure 4.4B). Similar to that observed with GR IHC, nuclear RelA protein levels were lower in SerCa compared to all
Table 4.4. Genes downregulated by GR showing increased expression in FTEb(S) samples.

<table>
<thead>
<tr>
<th>Probe Set ID</th>
<th>Gene Title</th>
<th>Symbol</th>
<th>Fold Change*</th>
</tr>
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<tbody>
<tr>
<td>222162_at</td>
<td>ADAM metallopeptidase with thrombospondin type 1 motif, 1</td>
<td>ADAMTS1</td>
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<td>207614_at</td>
<td>cullin 1</td>
<td>CUL1</td>
<td>1.41</td>
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<tr>
<td>201324_at</td>
<td>epithelial membrane protein 1</td>
<td>EMP1</td>
<td>1.75</td>
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<tr>
<td>227140_at</td>
<td>inhibin, beta A</td>
<td>INHBA</td>
<td>1.46</td>
</tr>
<tr>
<td>212298_at</td>
<td>neurophin 1</td>
<td>NRP1</td>
<td>3.13</td>
</tr>
<tr>
<td>209193_at</td>
<td>pim-1 oncogene</td>
<td>PIM1</td>
<td>3.05</td>
</tr>
<tr>
<td>209723_at</td>
<td>serpin peptidase inhibitor, clade B (ovalbumin), member 9</td>
<td>SERPINB9</td>
<td>3.03</td>
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**Cell Growth & Apoptosis (7)**

<table>
<thead>
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<th>Gene Title</th>
<th>Symbol</th>
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<td>201012_at</td>
<td>annexin A1</td>
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<td>201891_at</td>
<td>beta-2-microglobulin</td>
<td>B2M</td>
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<td>204905_at</td>
<td>B-cell CLL/Lymphoma 3</td>
<td>BCL3</td>
<td>5.00</td>
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<tr>
<td>212067_at</td>
<td>complement component 1, r subcomponent</td>
<td>C1R</td>
<td>8.49</td>
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<tr>
<td>218598_at</td>
<td>chemokine (C-C motif) ligand 2</td>
<td>CCL2</td>
<td>2.48</td>
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<tr>
<td>224833_at</td>
<td>v-ets erythroblastosis virus E26 oncogene homolog 1</td>
<td>ETS1</td>
<td>2.26</td>
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<tr>
<td>204472_at</td>
<td>GTP binding protein overexpressed in skeletal muscle</td>
<td>GEM</td>
<td>3.52</td>
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<td>202639_at</td>
<td>intercellular adhesion molecule 1</td>
<td>ICAM1</td>
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<td>interleukin 4 receptor</td>
<td>IL4R</td>
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<td>226333_at</td>
<td>interleukin 6 receptor</td>
<td>IL6R</td>
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<tr>
<td>202859_x_at</td>
<td>interleukin 5</td>
<td>IL8</td>
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**Inflammation/Immune Response (18)**

<table>
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<th>Gene Title</th>
<th>Symbol</th>
<th>Fold Change*</th>
</tr>
</thead>
<tbody>
<tr>
<td>203708_at</td>
<td>phosphodiesterase 4B, cAMP-specific (phosphodiesterase E4 ducne homolog)</td>
<td>PDE4B</td>
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<td>polo-like kinase 2</td>
<td>PLK2</td>
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<td>225973_at</td>
<td>transporter 2, ATP-binding cassette, sub-family B</td>
<td>TAP2</td>
<td>2.46</td>
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<tr>
<td>214581_x_at</td>
<td>tumor necrosis factor receptor superfamily, member 21</td>
<td>TNFRSF21</td>
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<tr>
<td>210512_s_at</td>
<td>vascular endothelial growth factor</td>
<td>VEGF</td>
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**Regulation of Transcription (8)**

<table>
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<tr>
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<th>Gene Title</th>
<th>Symbol</th>
<th>Fold Change*</th>
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</thead>
<tbody>
<tr>
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<td>BACH1</td>
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<tr>
<td>201170_s_at</td>
<td>basic helix-loop-helix domain containing, class B, 2</td>
<td>BHLHB2</td>
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<tr>
<td>208357_at</td>
<td>Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2</td>
<td>CITED2</td>
<td>1.56</td>
</tr>
<tr>
<td>204512_at</td>
<td>human immunodeficiency virus type 1 enhancer binding protein 1</td>
<td>HIVEP1</td>
<td>1.24</td>
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<tr>
<td>213147_at</td>
<td>homeobox A10</td>
<td>HOXA10</td>
<td>1.14</td>
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<tr>
<td>201437_at</td>
<td>jun B proto-oncogene</td>
<td>JUNB</td>
<td>1.85</td>
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<td>221559_s_at</td>
<td>lymphoid enhancer-binding factor 1</td>
<td>LEF1</td>
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<tr>
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<td>nuclear receptor subfamily 4, group A, member 2</td>
<td>NR4A2</td>
<td>1.52</td>
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<tr>
<td>Probe Set ID</td>
<td>Gene Title</td>
<td>Symbol</td>
<td>Fold Change</td>
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<tr>
<td>208818_s_at</td>
<td>catechol-O-methyltransferase</td>
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<tr>
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<td>degenerative spermatocyte homolog 1, lipid desaturase</td>
<td>DEGS1</td>
<td>1.64</td>
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<td>CXCR7</td>
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<td>HBEGF</td>
<td>2.02</td>
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<td>apolipoprotein A-II</td>
<td>APOA2</td>
<td>1.35</td>
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<td>217772_s_at</td>
<td>mitochondrial carrier homolog 2</td>
<td>MTCH2</td>
<td>1.14</td>
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<tr>
<td>203504_s_at</td>
<td>ATP-binding cassette, sub-family A (ABC1), member 1</td>
<td>ABCA1</td>
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<td>arrestin domain containing 3</td>
<td>ARRD3C</td>
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<td>protein tyrosine phosphatase type IVA, member 2</td>
<td>PTP4A2</td>
<td>1.36</td>
</tr>
<tr>
<td>217977_at</td>
<td>selenoprotein X, 1</td>
<td>SEPX1</td>
<td>1.97</td>
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<tr>
<td></td>
<td>serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin),</td>
<td></td>
<td></td>
</tr>
<tr>
<td>211429_at</td>
<td>member 1</td>
<td>SERPINA1</td>
<td>2.54</td>
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<tr>
<td>204079_at</td>
<td>tyrosylprotein sulfotransferase 2</td>
<td>TPST2</td>
<td>1.19</td>
</tr>
</tbody>
</table>

* corresponds to the relative expression of the specific probe set in FTEb(S) samples compared to FTEb(N) (decreased expression <0, increased expression >0). Italicized probe sets also show increased expression in SerCa relative to FTEb(N) samples.
FIGURE 4.3: Increased expression of RelA mRNA in FTEb(S) samples. Analysis of gene expression profiles revealed an increased expression of RelA in FTEb luteal relative to FTEb follicular samples (A). RelA mRNA was also found to be elevated in FTEb(S) compared to FTEn, FTEb(N) and SerCa (B), as well as other luteal samples (C). Different letters shown in A and B indicate statistically significant differences in average RelA mRNA expression (as determined by one-way ANOVA followed by Newman-Keuls Multiple Comparison post-hoc test; p < 0.05).
FIGURE 4.4: Increased expression of nuclear and cytoplasmic RelA protein in FTEb(S) samples. IHC on tissue microarrays revealed a trend towards increased average nuclear ('N') histoscore (A) and proportion of cases with a nuclear percentage score ≥2 (B) in FTEb(S) compared to FTEb(N) and FTEn samples. Similarly, there was a trend towards increased average cytoplasmic ('C') histoscore in FTEb(S) relative to FTEb(N) samples (C), and an increased proportion of FTEb(S) samples were given a cytoplasmic percentage score of ≥2 (D). Finally, decreased nuclear (A-B) and cytoplasmic (C-D) immunopositivity for RelA was observed in SerCa relative to non-malignant FTE samples. Representative staining for RelA in FTEb(N) (E), FTEb(S) (F) and SerCa (G) samples is shown. Both FTEb(N) and FTEb(S) samples showed intense nuclear and cytoplasmic staining in secretory (solid arrows), but not ciliated (dashed arrows), FTE cells. Statistically significant differences in RelA protein expression are indicated by different letters (as determined by one-way ANOVA followed by Newman-Keuls Multiple Comparison post-hoc test; p < 0.05).
non-malignant FTE groups, with an average nuclear histoscore of 2.17±0.20 and only 8/47 (17%) of cases showing a percentage score ≥2 (Figures 4.4A-B).

Unlike nuclear RelA protein, cytoplasmic positivity was observed in the majority of epithelial cells in each case examined. Similar to nuclear RelA, there was a trend towards increased average cytoplasmic histoscore in FTEb(S) (5.50±0.29) compared to FTEb(N) (4.63±0.88) samples (Figure 4.4C). FTEn samples showed an average cytoplasmic histoscore intermediate to FTEb(N) and FTEb(S) samples (5.17±0.32). Consistently, a cytoplasmic percentage score ≥2 (corresponding to >80% positivity) was observed in 4/4 (100%) of FTEb(S), 5/8 (62.5%) of FTEb(N) and 10/12 (83.3%) of FTEn samples (Figure 4.4D). Similar to nuclear staining, decreased cytoplasmic RelA expression was observed in SerCa samples relative to non-malignant FTE, with an average cytoplasmic histoscore of 3.19±0.29 and 18/47 (38%) of cases showing a percentage score ≥2 (Figures 4.4C-D). Representative staining for FTEb(N), FTEb(S) and SerCa samples is shown in Figures 4.4E-G. Of particular note is the intense nuclear and cytoplasmic RelA staining consistently observed in secretory rather than ciliated FTE cells within most samples. Altogether, FTEb(S) samples showed an increased expression of RelA mRNA, and (consistently) a trend towards increased percentage of positive cells for both nuclear and cytoplasmic RelA protein, relative to FTEb(N) samples.

4.4.4. Altered expression of inflammatory mediators in SerCa and select FTE

Initial analysis of gene expression profiles had revealed the altered expression of a surprisingly large number of genes involved in inflammation and/or immune response in FTEb(S) and FTEb luteal samples overall (see Appendices I and II). To explore the expression of these genes in more detail, a list of 144 genes (258 probe sets) positively regulated by NFκB was compiled in collaboration with bioinformatics specialist Carl Virtanen (146, 247-250). Clustering using this list separated FTEb(S) and SerCa from FTEb(N) samples (Figure 4.5), implicating altered inflammatory signalling during the luteal phase as a risk factor for malignant transformation. Interestingly, three FTEn luteal samples not previously shown to group with
FIGURE 4.5: Altered expression of inflammatory mediators in SerCa and select FTE.

Clustering using a cassette of probe sets corresponding to NFκB-induced genes confirmed the separation of FTEb(S) (boxed) from FTEb(N) samples. The cluster tree is shown at top, with each line representing one sample. The type of sample is indicated at the bottom (yellow, FTEn; red, FTEb; blue, SerCa), while the stage of the ovarian cycle for FTE samples is indicated below (purple, follicular; green, luteal). A portion of the heatmap is shown here, with the gene represented by the specific probe set in each row indicated at right.
SerCa also clustered with FTEb(S) samples using this cassette. Overall, 7/12 (58.3%) luteal and 0/12 follicular samples grouped with SerCa according to expression of NFκB-dependent inflammatory genes (p=0.0046).

To determine which probe sets were contributing to this clustering, RMA expression values for all probe sets were compiled. Prior to filtering for level of expression, 60/258 (23.3%) of the probe sets on the cassette were unaltered in FTEb(S) vs. FTEb(N) samples (fold change <10%), while 156/258 (60.5%) and 42/258 (16.3%) showed increased or decreased expression in FTEb(S) respectively. Of the genes showing altered expression in FTEb(S), 156/198 (78.8%) showed increased expression while 42/198 (21.2%) showed decreased expression compared to FTEb(N).

When probe sets showing background levels of expression were eliminated, 13/112 (11.6%) were unaltered, while 78/112 (69.6%) were increased and 21/112 (18.8%) were decreased in FTEb(S) samples. Of the filtered probe sets showing altered expression in FTEb(S), 78/99 (78.8%) showed increased expression while 21/99 (21.2%) showed decreased expression compared to FTEb(N). Interestingly, 75/99 (75.8%) of these altered probe sets showed the same direction of change in the three FTEn luteal samples grouping with FTEb(S) (relative to FTEb(N)). Specific genes upregulated by NFκB showing increased expression in FTEb(S) are highlighted in Table 4.5. Notably, 57/78 (73.1%) of these probe sets also showed increased expression in SerCa compared to FTEb(N) samples. Overall, FTEb(S) samples showed altered expression of a large proportion of NFκB-induced genes, in addition to GR-repressed genes implicated in inflammatory signalling. Similar relative expression of many of these genes in SerCa samples relative to FTEb(N) suggests that altered expression of GR- and NFκB-influenced genes contributes to the similarity of FTEb(S) and SerCa specimens.
Table 4.5. Genes upregulated by NFκB showing increased expression in FTEb(S) samples.

<table>
<thead>
<tr>
<th>Probe Set ID</th>
<th>Gene Title</th>
<th>Symbol</th>
<th>Fold Change*</th>
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<td>B2M</td>
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<td>CCND3</td>
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* corresponds to the relative expression of the specific probe set in FTEb(S) samples compared to FTEb(N) (decreased expression < 0, increased expression > 0). Italicized probe sets also show increased expression in SerCa relative to FTEb(N) samples.
4.5: DISCUSSION

The post-ovulatory luteal phase is characterized by an elevation of inflammatory cytokines at the ovarian surface (and presumably adjacent distal FTE), leading to activation of NFκB signalling and a compensatory anti-inflammatory response by GR. To determine if altered inflammatory signalling during the luteal phase may contribute to an increased propensity for malignant transformation, I characterized the expression of GR, NFκB and their respective targets in SerCa and non-malignant FTE during the luteal and follicular phase. Analysis of gene expression profiles revealed an elevated expression of GR mRNA in FTEb luteal samples, most notably those previously found to group with SerCa by unsupervised clustering. Expression in FTEb(S) samples was similar to that observed in SerCa, and both groups showed increased GR mRNA relative to FTEn, FTEb(N) and remaining luteal samples. Activation of GR signalling through ligand stimulation leads to downregulation of GR mRNA and protein levels in most cell types (145, 160, 251, 252). We therefore postulated that the observed increase in GR levels in FTEb(S) and SerCa samples may be reflective of disrupted GR signalling.

In the absence of ligand, GR monomers reside in the cytoplasm in an inactive state through association with heat shock proteins and other chaperones. Upon ligand binding, activated GR proteins dissociate from co-chaperone proteins, homodimerize and translocate into the nucleus to regulate transcription (145, 157-159). IHC was performed to determine whether FTEb(S) and SerCa samples showed increased expression and/or a predominately cytoplasmic localization of GR protein. While negligible cytoplasmic staining was present in all groups, nuclear positivity was observed in both secretory and ciliated FTE and carcinoma cells. In addition, FTEb(S) samples showed a trend towards an increased average nuclear histoscore, and an increased percentage of cells positive for nuclear GR staining, compared to FTEn and FTEb(N) samples. Overall, FTEb(S) samples showed an increased expression of GR mRNA and percentage of positive cells for nuclear GR protein compared to FTEb(N),
potentially reflective of disrupted GR signalling in these samples. Interestingly, despite similar levels of GR mRNA in SerCa and FTEb(S) samples, SerCa samples had a lower average nuclear histoscore and percentage of positive staining compared to all non-malignant FTE groups.

To my knowledge, this is the first comprehensive evaluation of GR expression in human fallopian tube specimens. GR expression has also been poorly characterized in clinical ovarian cancer samples to date; only a few studies have been performed with little detail provided. The first such study consisted of 10 patients (including 5 diagnosed with serous carcinoma) and reported predominately nuclear staining in 10-80% of tumour cells, although no further details were provided (258). Similarly, a second study of 85 patients (48 serous) reported a nuclear expression pattern for GR protein. Expression of GR had no prognostic impact in their study, and no details of relative staining by histotype, grade, or other clinical parameters were provided (259). A recent study of breast carcinoma reported that 25/25 (100%) of neoplastic cells within DCIS and LCIS and 249/254 (98%) of invasive, non-metaplastic carcinomas were negative for GR protein by IHC (260). An additional study reported that the percentage of patients with nuclear GR immunopositivity decreased with tumour development, with concomitant accumulation in the cytoplasm (261). Interestingly, a separate study of 25 patients reported an increased expression of GR mRNA in grade 3 breast carcinomas relative to grade 1/2 and benign tumours (262). The authors suggested that an unidentified pro-tumorigenic mechanism may exist that prevents the translation of GR mRNA in high grade specimens. It is also possible that the observed GR could be expressed as an alternate isoform, as the methods employed in their study (and ours) were unable to distinguish between GRα and the constitutively nuclear, transcriptionally inactive GRβ (145, 158, 160). It is unclear if either of these mechanisms could explain the decreased GR protein observed in SerCa specimens in the present study.
To further explore the hypothesis that GR signalling was specifically disrupted in FTEb(S) samples, expression profiles were re-assessed using directed gene-specific analysis to determine the expression of GR- influenced genes. Relative expression in FTEb(S) compared to FTEb(N) samples in the opposite direction as that typically observed in glucocorticoid-exposed cells would further suggest that these specific luteal samples were characterized by disrupted GR signalling. Consistently, FTEb(S) samples showed decreased expression of 20/55 (36.4%) of GR-activated genes, with the functional categories of regulation of transcription and signal transduction showing the greatest proportion of decreased genes. Of the genes outlined in Table 4.2, only AKAP13 (263), SNAI2 (264) and TGFBR3 (265) have been investigated in epithelial ovarian cancer in general, while none of the 20 genes have been specifically examined in ovarian SerCa or human fallopian tube epithelium and may represent novel tumour suppressor genes.

In addition to altered expression of a subset of GR-activated genes, FTEb(S) samples showed increased expression of a large proportion (43/62, 69.4%) of genes typically repressed by GR, most notably 80% of genes involved in inflammation and/or immune response. Five of these inflammatory genes outlined in Table 4.4 (CCL2, ETS1, ICAM1, TAP2 and VEGF) have previously been implicated in epithelial ovarian cancer (266-271). Interestingly, pro-angiogenic vascular endothelial growth factor (VEGF) has previously been found to be secreted by human FTE primary cultures following stimulation with the inflammatory cytokine TNFα (272). Ciliated and secretory FTE cells also express intercellular adhesion molecule 1 (ICAM1) along the luminal border of the human fallopian tube; the expression of this gene has similarly been shown to be induced by inflammatory cytokines in primary culture (273). Finally, primary human FTE cultures constitutively produce interleukin-8 (IL-8) and CCL2, potent chemokines for neutrophils and monocytes respectively (155, 274). The presence of IL8 protein has also been demonstrated in human FTE by IHC, with the greatest positivity observed at the apical membrane in the distal compared to proximal tube during the peri-ovulatory period (275).
The GR inhibits inflammatory signalling primarily through inhibition of NFkB activity. Proposed mechanisms for this inhibition have included but are not limited to physical interaction of GR and RelA proteins, competition for cofactors such as CBP and interference with phosphorylation of RNA polymerase II at promoters of NFkB target genes (145, 149, 150, 161, 163, 164). GR and RelA are mutually inhibitory, as RelA can similarly decrease GR-mediated transcription (145, 148). Given our findings suggestive of disrupted GR signalling in FTEb(S) samples, we hypothesized that NFkB-dependent signalling may also be altered in these same samples.

We first set to determine the expression of RelA mRNA and protein in profiled samples. Analysis of gene expression profiles revealed an increased expression of RelA mRNA in FTEb luteal relative to FTEb follicular samples, as well as in FTEb(S) samples relative to FTEn, FTEb(N) and the remaining luteal samples. Unlike GR mRNA, RelA was not similarly elevated in SerCa specimens, suggesting that overexpression of RelA may be exclusively involved in the predisposition and/or initial events of serous carcinogenesis.

In the absence of ligand, monomers of NFkB subunits reside in the cytoplasm in an inactive state through association with IκB inhibitory proteins. Upon ligand stimulation by inflammatory cytokines such as TNFα and IL-1, IκB proteins are promptly phosphorylated and degraded, leading to subunit release and translocation to the nucleus where (primarily) RelA-p50 heterodimers can activate transcription (145, 147, 151). IHC was therefore performed for RelA, with the hypothesis that FTEb(S) samples would express increased levels of nuclear RelA protein and/or diminished cytoplasmic positivity consistent with enhanced NFkB activity. As hypothesized, I observed a trend towards an increased nuclear histoscore and percentage of positive cells in FTEb(S) compared to FTEn and FTEb(N) samples, likely reflecting an enhanced capability to activate NFkB target genes. Contrary to what was hypothesized, all fallopian tube specimens examined showed cytoplasmic RelA positivity in the majority of epithelial cells. In addition, there was a trend towards increased cytoplasmic histoscore and percentage of positive cells in FTEb(S) relative to FTEb(N) samples, with FTEn samples
showing intermediate expression. Although cytoplasmic NFκB would be incapable of activating target gene transcription, previous studies have demonstrated a necessary cytoplasmic component for RelA-GR cross-repression (150). In fact, RelA mutants missing the nuclear translocation signal were capable of inhibiting hormone-dependent GR transcription equivalent to wildtype RelA protein. This inhibition was found to be dependent on cytoplasmic PKA-induced phosphorylation of the Ser276 residue of RelA (150). It is therefore possible that the increased cytoplasmic RelA observed in FTEb(S) is, at least in part, reflective of RelA capable of inhibiting GR-dependent activity rather than accumulation of inactive RelA subunits.

The most striking finding of RelA IHC was the intense nuclear and cytoplasmic staining consistently observed in secretory rather than ciliated FTE cells. This expression pattern was observed in both luteal and follicular phase samples, with an increased percentage of positive cells in the luteal phase (likely in part due to the increased proportion of secretory cells during this phase). As previously discussed (Section 1.12.5), secretory FTE cells are a proposed cell-type of origin for high grade SerCa. Of particular interest, we have previously reported a specific loss of the tumour suppressor DAB2 in this cell-type during the luteal phase (Chapter 2). The opposite expression patterns observed in secretory cells during the luteal phase may suggest an inverse relationship between these two proteins, which will be explored in the following chapter.

Similar to that observed with GR IHC, SerCa showed lower levels of both nuclear and cytoplasmic RelA protein compared to all non-malignant FTE groups, for reasons that are unclear. Two recent studies have evaluated RelA protein expression in clinical ovarian cancer samples. The first study reported RelA immunopositivity in 36/50 (72%) of epithelial ovarian cancers, compared to 6/20 (30%) of ovarian benign adenomas and 1/10 (10%) of normal tissues. Expression of RelA protein was found to correlate with tumour stage and grade, but not histological type (276). The second study described elevated expression of RelA in epithelial ovarian cancer (including 31/40 serous tumours) compared to normal ovarian tissue, with high RelA being significantly correlated with late clinical stage and grade (277). Both nuclear and
cytoplasmic RelA staining was observed, however it was not indicated whether these were separately scored. Distinct criteria for positivity and the use of different reference tissue (normal ovary vs. fallopian tube epithelium) compared to my study may partly explain the discrepancies observed. More importantly, it is critical to assess nuclear RelA staining independently as this is more reflective of transcriptionally active NFκB.

To determine if altered expression of NFκB target genes contributes to the similarity of FTEb(S) and SerCa samples, cluster analysis was performed using a list of 258 probe sets corresponding to 144 NFκB-induced genes. Clustering using this list successfully separated FTEb(S) and SerCa from FTEb(N) samples, implicating altered inflammatory signalling during the luteal phase as a potential risk factor for malignant transformation. Interestingly, three FTEn luteal samples also grouped with FTEb(S) using this cassette of genes. Re-evaluation of the clinical history of these three women did not reveal any obvious commonalities that could distinguish them from the other normal control patients, suggesting that more in depth molecular characterization of these samples is warranted.

To determine which specific NFκB target genes could be contributing to these cluster results, RMA expression values for all probe sets were compiled. After filtering for level of expression, FTEb(S) samples showed increased expression of 78/99 (78.8%) altered probe sets (corresponding to 60 unique NFκB target genes). Of the genes outlined in Table 4.5, 14 (including BGN, CCL2, CXCL1, ICAM1, IGFBP1, IL15, IL1RN, LCN2, MMP1, MYC, SOD2, THBS1, THBS2 and VEGFC) have previously been implicated in epithelial ovarian cancer (154, 266, 267, 269, 278-287). Four genes increased in FTEb(S) – namely SOD2, VCAM1, CTSB and IL15 – were recently found to be elevated in inflammatory vs. non-inflammatory breast cancer (146). Only one gene, insulin-like growth factor binding protein 1 (IGFBP1), has been previously observed in human fallopian tube samples. Immunopositivity for IGFBP1 was localized to the cytoplasm of FTE cells, with increased mRNA and protein expression observed following treatment with mifepristone (288). Expression of IGFBP1 has also been positively associated with risk of ovarian cancer in a case-control study (280).
Overall, the gene expression patterns observed in FTEb(S) samples is consistent with altered inflammatory signalling in the distal fallopian tube in a subset of luteal phase samples. In fact, these samples show enhanced expression of chemokines responsible for recruitment of inflammatory leukocytes and genes contributing to intracellular survival signalling. In addition to CCL2 and IL8 discussed above, FTEb(S) samples showed increased expression of CXCL1, CXCL2 and CXCL3 (also known as Gro-α, Gro-β and Gro-γ), related chemokines responsible for recruitment of neutrophils to sites of inflammation (289, 290). Previous studies have observed large numbers of neutrophils in human fallopian tube specimens, including some within the epithelial compartment itself (291, 292). Neutrophils are the first cells to migrate into inflammatory sites, where they eliminate pathogens by phagocytosis, release of microbicidal compounds from intracellular granules and produce toxic oxidative compounds (291). Compounds released by neutrophils and other inflammatory leukocytes can also damage nearby cells. In addition to chemokines, FTEb(S) samples express increased levels of superoxide dismutase 2 mitochondrial (SOD2), a gene induced by inflammatory cytokines and oxidative damage which acts to convert superoxide radicals to hydrogen peroxide (154). SOD2 acts mainly as a survival protein, by maintaining mitochondrial integrity in cells exposed to adverse conditions and preventing induction of p53-dependent apoptosis (293). Proponents of the theory that ovulation is akin to an acute inflammatory event have hypothesized that survival of OSE cells with inflammation-induced sublethal damage adjacent to the ovulatory site contributes to ovarian carcinogenesis (76, 90, 91, 102, 154). Interestingly, recent studies have observed expression of the DNA damage marker γ-H2AX in 20/30 (66%) of p53 signatures in the distal fallopian tube (198). Experiments should therefore be performed to determine the direct effect of ovulation-associated inflammatory mediators on adjacent distal FTE cells.

In this study, a large proportion of NFκB target genes showed increased expression in 7/12 luteal samples obtained from both BRCA1 mutation carriers and controls. Intriguingly, one previous study reported a co-activator role for BRCA1 in NFκB transcriptional activity (294). Transient transfection with wild-type BRCA1 augmented both TNFα and IL-1β-dependent
transcription of the NFκB target genes *Fas* and *interferon-β*. This effect was dependent on the interaction of endogenous BRCA1 and RelA proteins, and phosphorylation of RelA at Ser276. The effect of BRCA1 on global NFκB-dependent transcription was not determined. Interestingly, FTEb(S) and the closely associated FTEn luteal samples showed similarly increased expression of *Fas*, also known as *tumor necrosis factor receptor superfamily member 6* (*TNFRSF6*), in comparison to FTEb(N) samples. This example illustrates the difficulty of determining the individual contributions of *BRCA* mutation status and the luteal-phase milieu in altered expression of NFκB target genes.

In summary, post-ovulatory FTEb(S) samples show gene expression profiles consistent with disrupted GR signalling and elevated inflammatory signalling. Specifically, these samples show altered expression of GR and the active NFκB subunit RelA, as well as increased expression of a large proportion of NFκB-induced genes and GR-repressed genes implicated in inflammatory signalling. Similar relative expression of many of these genes in SerCa samples relative to FTEb(N) suggests that altered expression of GR- and NFκB-influenced genes contributes to the similarity of FTEb(S) and SerCa specimens. Finally, similar altered expression of NFκB targets in a subset of luteal phase samples obtained from normal controls suggests that factors in addition to *BRCA* mutation status and the luteal phase milieu may be involved.
CHAPTER 5

DAB2 ACTIVATES GLUCOCORTICOID RECEPTOR-MEDIATED ANTI-INFLAMMATORY SIGNALLING IN OVARIAN CANCER CELLS

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This manuscript is currently in preparation for submission.

NOTES:

I completed all of the work presented in this chapter.
5.1: ABSTRACT

Purpose: We previously demonstrated decreased expression of DAB2, an adaptor protein implicated in AR and growth factor signalling pathways, in SerCa and FTEb during the post-ovulatory luteal phase. Our data also indicated diminished GR signalling and an elevated expression of pro-inflammatory genes in DAB2-deficient samples. The objective of this study was to determine whether DAB2 is also involved in regulation of anti-inflammatory signalling by GR.

Experimental Approach: To determine the effect of DAB2 on GR transactivation activity, glucocorticoid-responsive ES2 ovarian cancer cells were co-transfected with a DAB2 expression construct and a GR-responsive luciferase reporter gene. Cells were treated with 10nM dexamethasone (dex) or vehicle and harvested 24h later for luciferase activity determination. To determine the impact of DAB2 on GR transrepression of NFκB, cells were co-transfected with DAB2-specific siRNA and an NFκB-responsive luciferase reporter, treated with 10ng/mL of the ovulation-associated cytokine TNFα and/or 100nM dex, and harvested 8h later for luciferase activity determination. To determine if DAB2 alters mRNA expression of RelA or its targets, cells were transfected with DAB2 siRNA, treated with dex and/or TNFα and harvested for total RNA extraction and RT-qPCR. Co-immunoprecipitation studies were performed to determine if DAB2 interacts with GR or RelA.

Results: DAB2 overexpression enhanced dex-induced GR transactivation activity relative to empty vector. Conversely, DAB2-specific siRNA increased TNFα-induced NFκB activity relative to non-targeting siRNA, suggesting that DAB2 also enhances GR-mediated transrepression. DAB2 siRNA increased mRNA levels of NFκB target genes SOD2 and cathepsin B (CTSB) in vehicle- and/or dex-treated cells, while no effect was observed for vascular cell adhesion molecule 1 (VCAM1). DAB2 siRNA combined with TNFα treatment led to potent induction of SOD2, but not CTSB or VCAM1, mRNA levels. While DAB2 did not significantly impact RelA mRNA levels in vehicle-, dex- or TNFα-treated cells, endogenous DAB2 protein was found to
interact with both GR and RelA in vehicle- and dex-treated cells. This suggests that DAB2 may impact GR-mediated suppression of NFκB signalling in part via protein-protein interactions.

**Conclusions:** These data support a role for DAB2 in activation of GR-mediated transactivation and suppression of NFκB signalling. We therefore postulate that the decreased DAB2 expression and GR signalling observed in select luteal phase FTE may result in increased pro-inflammatory signalling in these samples, and may play a key role in promoting serous carcinogenesis.
**5.2: INTRODUCTION**

We have previously demonstrated decreased mRNA and cytoplasmic protein expression of the tumour suppressor DAB2 in SerCa and FTEb during the post-ovulatory luteal phase (Chapter 2), including those grouping with SerCa by unsupervised clustering. DAB2 was originally identified in a screen for transcripts downregulated in ovarian cancer cell lines compared to normal OSE cells (295). Subsequent studies have observed decreased DAB2 in breast, prostate, pancreatic and esophageal cancer (296, 297). DAB2 has been implicated in various cellular processes, including proliferation, differentiation, polarity, endocytosis, adhesion and migration (298-303). It has also been shown to regulate important signalling pathways, most notably the AR pathway (298), canonical and non-canonical TGFβ signalling (214, 300, 303), AP-1 signalling (304-306) and the canonical Wnt pathway (297). DAB2 primarily functions as an adaptor protein within these pathways, with an N-terminal phosphotyrosine-binding domain previously shown to associate with transmembrane proteins, and a C-terminal proline-rich domain capable of interacting with SH3 domain-containing signaling molecules (222).

Interestingly, the same subsets of samples with decreased DAB2 show evidence of diminished GR signalling, characterized by an elevation of GR mRNA and altered expression of both GR-induced and GR-repressed genes. At the same time, these samples show evidence of elevated NFκB-dependent inflammatory signalling, characterized by increased expression of the active NFκB subunit RelA and a large number of NFκB target genes (Chapter 4). Furthermore, during the luteal phase secretory FTE cells show intense immunopositivity for RelA and conversely specific loss of cytoplasmic DAB2 protein.

These observations, combined with the known inhibition of the steroid hormone receptor AR by DAB2, led us to hypothesize that DAB2 may also regulate signalling by GR, including repression of RelA. The purpose of this study was therefore to investigate the impact of DAB2 on GR transactivation and transrepression activity in a glucocorticoid-responsive cell culture model, and to begin to understand the potential mechanisms involved.
5.3: MATERIALS AND METHODS

5.3.1. Cell culture

ES2 and SKOV3 ovarian cancer cells were grown in RPMI 1640 medium, supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, 100μg streptomycin and fungizone (all purchased from Gibco Cell Culture Products/Invitrogen). BT20 and MCF7 breast cancer cells were grown in DMEM/F-12 medium (Gibco) supplemented as described above. Medium containing charcoal-stripped, heat-inactivated FBS was used during experiments requiring hormone treatments. The synthetic glucocorticoid dexamethasone (dex) was dissolved in ethanol, whereas recombinant human TNFα (R&D Systems) was reconstituted in sterile PBS and 0.1% BSA. Dex and TNFα were diluted with culture medium before addition to cell cultures. Vehicle treatment consisted of an equivalent amount of ethanol diluted in culture medium, and all treatments were 24h unless otherwise specified.

5.3.2. Expression constructs and siRNA reagents

Expression constructs for the GR-responsive mouse mammary tumor virus (MMTV) luciferase reporter gene, β-galactosidase and pcDNA3 were obtained from Dr. Alex Kollara (University of Toronto). An expression vector (pOTB7) containing full-length DAB2 was obtained from Dr. Katherine Sodek (University of Toronto), and was inserted into the pcDNA3.1/Hygro vector by double digestion with NheI and XhoI followed by QIAquick gel extraction (Qiagen). The pNFκB-luc and (negative control) pTAL-Luc vectors were purchased from Clontech. All expression constructs were grown in DH5α bacteria and sequence verified. siGENOME SMARTpool DAB2-specific siRNA (containing a pool of four individual DAB2-targeting siRNAs) and non-targeting siRNA were purchased from Dharamacon and reconstituted in 1X siRNA buffer.
5.3.3. Luciferase assays

To test for endogenous GR transactivation activity, ES2, MCF7 and BT20 cells were plated at 100,000 cells/well in 24-well plates and transiently transfected with 0.1µg/well each of MMTV luciferase and β-galactosidase 24h later using Lipofectamine LTX (Invitrogen) according to the manufacturer’s recommended protocol. The total amount of plasmid DNA/well was brought up to 0.8µg by addition of empty pcDNA3 vector. Cells were treated with 10nM dex or vehicle 24h following transfection, and harvested in 1X Reporter Lysis Buffer (Promega) 24h after treatment. Luciferase activity was determined using a luminometer following addition of Luciferase Assay Substrate (Promega), and was normalized to β-galactosidase activity (measured as described by Sambrook and Russel (307)) to account for potential differences in transfection efficiency. Normalized data are expressed as the fold change relative to vehicle treated cells.

To determine the impact of DAB2 on (endogenous) GR transactivation activity, ES2 cells were seeded at 100,000 cells/well in 24-well plates and transfected with MMTV luciferase, β-galactosidase and DAB2 (or pcDNA3) 24h later. Cells were treated with 10nM dex or vehicle 24h following transfection, harvested 24h after treatment and luciferase and β-galactosidase activity were determined as described above. Triplicate wells were included for each condition within each experiment, and the experiment was repeated three times. Normalized data are expressed as the average fold change (+/-SEM) relative to pcDNA3-transfected, vehicle-treated cells. Statistical analysis was performed using one-way ANOVA followed by Newman-Keuls Multiple Comparison post-hoc test.

To determine the impact of DAB2 on (endogenous) GR transrepression activity, ES2 cells were plated as described above and transfected with pNFκB-luc, β-galactosidase and 50pmol DAB2-specific (or non-targeting) siRNA 24h later. Cells were treated with 100nM dex and/or 10ng/mL TNFα 48h following transfection (to allow for maximal siRNA-mediated decrease of endogenous DAB2 protein levels). Dex was added to cells 1h prior to TNFα, and a higher concentration (100nM) was used compared to earlier experiments to ensure optimal
inhibition of NFκB activity. Cells were harvested 8h following transfection, and luciferase and β-galactosidase activity were determined as described above. The average (normalized) luciferase activity (+/- SEM) was calculated for each condition using triplicate wells. Preliminary statistical analysis was performed using one-way ANOVA followed by Newman-Keuls Multiple Comparison post-hoc test.

5.3.4. Western blotting

To characterize the expression of DAB2, GR and RelA in ovarian and breast cancer cell lines, vehicle-treated cells were lysed in RIPA lysis buffer containing Complete Protease Inhibitor Cocktail (Roche) and quantitated using the BCA Protein Assay (Pierce) according to the manufacturer’s recommended protocol. Aliquots of lysates containing 10μg of protein were resolved by 8% SDS-PAGE, and transferred to an Immobilon-P transfer membrane (Millipore). The membranes were preincubated for 1.5h in PBS containing 1% Tween-20 and 5% instant skim milk powder, followed by incubation with primary antibody for 2h. Antibodies used include: mouse monoclonal anti-DAB2/p96 (1:500; BD Transduction Laboratories), rabbit polyclonal anti-NFκB p65(A) (1:500), mouse monoclonal anti-GR (3D5) (1:50) and goat polyclonal anti-actin (1:500) (all from Santa Cruz Biotechnology). Immunoreactive proteins were visualized with HRP-labeled goat anti-mouse (DAB2, GR), goat anti-rabbit (NFκB) or donkey anti-goat (actin) antiserum for 1h (1:1500; Santa Cruz Biotechnology). Proteins were detected by ECL or ECL Plus (for GR) (Amersham Pharmacia Biotech) on HyBlot CL autoradiography film (Denville Scientific).

5.3.5. RT-qPCR

To confirm the functional downregulation of GR mRNA with dex treatment, vehicle and dex-treated ES2 cells were harvested in TRIzol reagent (Invitrogen) and RNA was isolated. Contaminating DNA was removed (DNA-free kit, Ambion), and first-strand cDNA was
generated using Superscript III Reverse Transcriptase and Oligo(dT)$_{20}$ primers (Invitrogen). Samples were diluted to 1.6ng/μL using sterile double distilled water and RT-qPCR was performed as described in Section 2.3.6. Primer sequences for GR included: forward 5’-TCAGTGATGGG-AGAGTAGATGGTG-3’ and reverse 5’-CCATCCTTTTTGACTGTGGAGAT-3’. GR mRNA expression (normalized to ACTB) is expressed as the average fold change relative to vehicle-treated cells (n=2 experiments).

To determine the impact of DAB2 on mRNA expression of RelA and select target genes, ES2 cells were plated at 100,000 cells/well in 24-well plates, followed by transfection with 50pmol DAB2-specific (or non-targeting) siRNA. Cells were treated with: a) 10nM dex or vehicle or b) 100nM dex and/or 10ng/mL TNFα 48 hours following transfection. Cells were harvested at: a) 24h or b) 8h following treatment in 250 μL TRIzol reagent, and the 2 wells/condition were pooled to increase yield. RNA extraction, DNase treatment, reverse transcription and RT-qPCR was then performed as previously described. Primer sequences used included: SOD2 forward 5’-AACAACAGGCGCTTATCCACTG-3’, SOD2 reverse 5’-ACGATCGTGTTTACTTTTGCG-3’, CTSB forward 5’-GCTTAACAAAGGTTACCATAAGCC-3’, CTSB reverse 5’-TGGGAGCAGGGAGAACTTTAT-3’, VCAM1 forward 5’-CTATACCATCCGAAAGGCCC-3’, VCAM1 reverse 5’-AGCACGAGAAGCTCAGGAGA-3’, RelA forward 5’-ATGGCCTCCTTTTCAGGAGAT-3’ and RelA reverse 5’-TCCGTAAGTGTCTTTGGAGG-3’. mRNA expression (normalized to ACTB) in cells treated for 24h (a) is expressed as the average fold change (+/-SEM) relative to vehicle-treated cells transfected with non-targeting siRNA (n=3 experiments). Statistical analysis was done by one-way ANOVA followed by Newman-Keuls Multiple Comparison test.

**5.3.6. Co-immunoprecipitation**

Vehicle- and dex-treated ES2 cells were lysed in EBC lysis buffer containing Complete Protease Inhibitor Cocktail (Roche). Following centrifugation, the supernatant was transferred to a fresh tube and incubated overnight at 4°C with anti-DAB2 antibody (Santa Cruz
Biotechnology) and for 1h at 4°C with Protein A-Sepharose 4B (Invitrogen) with constant mixing. Following centrifugation, the protein-bead mixture was washed five times with NETN containing 900mM NaCl, and washed once with NETN containing 100mM NaCl. The pellet was then resuspended in 2X-SDS loading dye and Western blotting (for GR and RelA) was performed as described above.

5.4: RESULTS

5.4.1. ES2 ovarian cancer cells have functional GR signalling

To investigate whether DAB2 regulates GR-dependent signalling a suitable cell culture model was required. Responsiveness to glucocorticoids, endogenous expression of our main proteins of interest (DAB2, GR, RelA), and efficiency of transfection were the criteria used to screen available cell lines. Efficiency of transfection by Lipofectamine LTX was first tested in various ovarian cancer (ES2, SKOV3) and breast cancer (BT20, MCF7) cell culture models. ES2 cells showed the greatest level of transfection (50-60%), with BT20 (25%), MCF7 (10-15%) and SKOV3 (<10%) cells showing lower levels (data not shown). High levels of transfection-induced cytotoxicity were observed in SKOV3 cells; therefore, this model was excluded. To confirm glucocorticoid responsiveness, the remaining cell culture models were transfected with a GR-responsive luciferase reporter gene (MMTV), treated with 10nM dex or vehicle, and harvested 24h later for determination of luciferase activity. MMTV consists of several tandem GREs, and is therefore a means to measure GRE-mediated gene expression (‘transactivation’) (254, 308). While MCF7 (2.1-fold, Figure 5.1A) and BT20 (1.3-fold, Figure 5.1B) cells showed low levels of dex-induced luciferase activity, ES2 cells (27.2-fold, Figure 5.1C) showed high levels of MMTV induction. To further confirm the suitability of ES2 cells in this study, Western blots were performed using vehicle-treated lysates of all cell lines. Figure 5.1D demonstrates the endogenous expression of GR, DAB2 and RelA proteins in ES2 (and SKOV3) cells, while other cell culture models only consistently showed the presence of RelA,
**FIGURE 5.1: ES2 ovarian cancer cells have functional GR signalling.** To test for glucocorticoid responsiveness, MCF7 (A), BT20 (B) and ES2 (C) cells were transfected with a GR-responsive (MMTV) luciferase reporter gene, treated with 10nM dex or ethanol vehicle and harvested 24h later for determination of luciferase activity. Only ES2 cells (C) showed high levels of luciferase induction with dex treatment. Luciferase activity (normalized to β-galactosidase) relative to vehicle-treated cells is shown in A-C. Western blotting confirmed the endogenous expression of DAB2, GR and RelA proteins in vehicle-treated ES2 cell lysates (D). RT-qPCR and Western blotting confirmed the functional downregulation of GR mRNA (E, n=2 experiments) and protein (F) in ES2 cells following 24h and 30h of dex treatment respectively. Both mRNA and protein expression shown in E and F are normalized to actin and expressed relative to vehicle-treated cells.
but not DAB2 or GR, proteins. Finally, the presence of functional GR signalling in ES2 cells was further confirmed by downregulation of GR mRNA (0.8-fold, Figure 5.1E) and protein (0.5-fold, Figure 5.1F) following 24h and 30h dex treatment respectively, using RT-qPCR and Western blotting. Because the ES2 cell line was the only one that fulfilled all of our criteria, all subsequent experiments in this chapter were performed using this model system.

5.4.2. DAB2 enhances GR transactivation and transrepression activity

In light of the altered expression of a subset of GR-induced genes in DAB2-deficient sample groups in the previous chapter, we set to determine whether DAB2 directly impacts GR transactivation activity. To test this, glucocorticoid-responsive ES2 cells were co-transfected with a DAB2 expression construct and MMTV-luciferase, treated with 10nM dex or vehicle and harvested 24h later for luciferase activity determination. In cells transfected with pcDNA3 vector only, dex treatment resulted in a 10.4-fold induction of luciferase activity relative to vehicle (p<0.001, Figure 5.2). This was increased to 18.6-fold and 26.5-fold induction of luciferase activity with dex treatment in cells transfected with 0.3μg or 0.5μg DAB2 respectively (relative to vehicle, both p<0.001). Overall, cells transfected with 0.5μg DAB2 showed a 2.5-fold enhanced induction of GR transactivation activity with dex treatment relative to empty vector (p<0.001). Levels of DAB2 protein in vehicle-treated cells transfected with empty vector or 0.5μg DAB2 (1.4-fold increase) are shown.

An additional observation in the previous chapter was the altered expression of a large proportion of GR-repressed genes and NFκB targets in DAB2-deficient sample groups. Since GR primarily exerts its anti-inflammatory effects through inhibition of NFκB activity, we set to determine whether DAB2 also impacts GR-mediated transrepression of NFκB. Hence, ES2 cells were co-transfected with DAB2-specific siRNA and an NFκB-responsive luciferase reporter, treated with 10ng/mL of the ovulation-associated cytokine TNFα and/or 100nM dex, and harvested 8h later for luciferase activity determination. In cells transfected with 50pmol of non-targeting siRNA control (‘NTsi), TNFα treatment led to a 2.9-fold induction of NFκB-
**FIGURE 5.2: DAB2 enhances GR transactivation activity.** ES2 cells were co-transfected with empty vector (pcDNA3) or a DAB2 expression construct and MMTV-luciferase, treated for 24h and luciferase activity determined to test if DAB2 directly impacts GR transactivation activity (n=3 experiments, triplicate wells each expt). Cells transfected with either 0.3μg or 0.5μg wild-type DAB2 showed enhanced luciferase induction compared to cells transfected with pcDNA3 only. Normalized data are expressed as the average fold change (+/- SEM) relative to pcDNA3 transfected/vehicle-treated cells. Statistically significant differences in luciferase activity are indicated by different letters (as determined by one-way ANOVA followed by Newman-Keuls Multiple Comparison post-hoc test; p < 0.05). Levels of DAB2 protein (as detected by Western blotting) in vehicle-treated cells transfected with pcDNA3 or 0.5μg DAB2 are shown at top right.
luciferase activity relative to vehicle (p<0.001, Figure 5.3). In addition, dex treatment decreased TNFα-induced (p<0.001), but not basal, NFκB activity. In cells transfected with 50pmol DAB2-specific siRNA (‘DAB2si’), TNFα treatment led to a 3.9-fold induction of luciferase activity relative to vehicle (p<0.001). Similar to NTsi, dex decreased TNFα-induced (p<0.001) but not basal NFκB activity. Overall, DAB2si enhanced NFκB-dependent luciferase activity (relative to NTsi control) within all treatment groups, with the greatest induction observed in TNFα-treated cells (2.2-fold). Similar induction by DAB2si was observed in vehicle-, dex- and TNFα+dex-treated cells (1.6-fold, 1.4-fold and 1.6-fold respectively). Levels of DAB2 protein in vehicle-treated cells following transfection with 50pmol of non-targeting or DAB2si (3.3-fold decrease) are shown. In summary, these results indicate that DAB2 enhances both GR transactivation activity and GR-mediated transrepression of NFκB in an ovarian cancer cell line.

5.4.3. DAB2 alters expression of select NFκB target genes

To determine if DAB2 alters mRNA expression of NFκB targets, cells were initially transfected with DAB2si, treated with dex or vehicle and harvested 24h later for total RNA extraction. RT-qPCR was then performed for SOD2, CTSB and VCAM1, genes found to be elevated in FTEb(S) samples in the previous chapter and also in a recent study of inflammatory breast cancer (146). No difference in SOD2 mRNA was observed in NTsi-transfected cells treated with dex vs. vehicle, while DAB2si-transfected cells showed 1.3-fold elevated SOD2 levels with dex treatment (p<0.05, Figure 5.4A). Within treatments, DAB2si resulted in a 1.7-fold increase in SOD2 mRNA in dex-treated cells (p<0.01), while no difference was observed in vehicle-treated cells. In addition, no difference in CTSB mRNA was observed in NTsi- or DAB2si-transfected cells treated with dex vs. vehicle (Figure 5.4B). Within treatments, DAB2si led to a 1.2-fold increase in CTSB mRNA levels in both vehicle- and dex-treated cells (p<0.05). Finally, no significant difference in VCAM1 mRNA was observed with either transfection of DAB2si or dex treatment (Figure 5.4C).
FIGURE 5.3: DAB2 enhances GR-mediated repression of NFκB activity. ES2 cells were co-transfected with non-targeting (NTsi) or DAB2-specific (DAB2si) siRNA and an NFκB-responsive luciferase reporter, treated with TNFα and/or dex for 8h and luciferase activity determined to test if DAB2 impacts GR transrepression activity (n=1 experiment, triplicate wells). Cells transfected with DAB2si showed enhanced luciferase activity (compared to NTsi-transfected cells) within all treatment groups, with the greatest induction observed in cells treated with the NFκB ligand TNFα. The average luciferase activity (normalized to β-galactosidase) was calculated for each condition using triplicate wells. Statistically significant differences in average luciferase activity are indicated by different letters (as determined by one-way ANOVA followed by Newman-Keuls Multiple Comparison post-hoc test; p < 0.05). Levels of DAB2 protein detected in vehicle-treated cells following transfection with NTsi and DAB2si are shown at top right.
FIGURE 5.4: DAB2 alters the expression of select NFκB target genes. ES2 cells were transfected with NTsi or DAB2si, treated with dex or vehicle for 24h, and RT-qPCR performed for SOD2, CTSS and VCAM1 (n=3 experiments). Both SOD2 (A) and CTSS (B) mRNA were increased by DAB2si (compared to NTsi) in dex-treated cells, whereas no effect on VCAM1 (C) was observed. In a separate experiment, ES2 cells were transfected with DAB2si (or NTsi), treated with dex and/or TNFα for 8h, and RT-qPCR performed for SOD2, CTSS and VCAM1 (n=1 experiment). Whereas SOD2 (D) mRNA levels were substantially increased by DAB2si in TNFα-treated cells, CTSS (E) levels were slightly increased and VCAM1 (F) levels were slightly decreased under the same conditions. mRNA expression (normalized to actin) relative to NTsi-transfected, vehicle-treated cells are shown in A-E. Different letters shown in A-C indicate statistically significant differences in average mRNA expression (as determined by one-way ANOVA followed by Newman-Keuls Multiple Comparison Test; p < 0.05).
Following my preliminary findings suggesting DAB2 affects TNFα-induced NFκB activity, ES2 cells were transfected with DAB2si, treated with dex and/or TNFα and harvested 8h later for RT-qPCR for SOD2, CTSB and VCAM1 (Figure 5.4D-F). In cells transfected with NTsi or DAB2si, both TNFα and TNFα+dex treatment increased SOD2 mRNA expression relative to vehicle, while dex treatment alone had no effect (Figure 5.4D). Within treatment groups, DAB2si increased SOD2 levels relative to NTsi in TNFα-, dex- and TNFα+dex-treated cells (1.9-fold, 1.3-fold and 1.4-fold respectively), while no difference was observed in vehicle-treated cells. Most notably, while TNFα treatment induced SOD2 mRNA levels by 4.2-fold in cells transfected with NTsi relative to vehicle, this was increased to 8.6-fold in DAB2si-transfected cells consistent with the observed effects on NFκB luciferase activity.

In contrast to SOD2, negligible effects on CTSB mRNA levels were observed in NTsi-transfected cells with TNFα (or dex) treatment alone, while a slight decrease was observed with combination TNFα+dex treatment relative to vehicle (Figure 5.4E). No treatment effects were observed for cells transfected with DAB2si. Within treatment groups, no change was observed with DAB2si in vehicle- and dex-treated cells, while CTSB mRNA was slightly increased in TNFα- (1.2-fold) and TNFα+dex-treated cells (1.3-fold).

Finally, in cells transfected with NTsi or DAB2si, VCAM1 mRNA levels were similarly increased with TNFα and TNFα+dex treatment relative to vehicle, while no effect was observed with dex treatment alone (Figure 5.4F). Within treatment groups, DAB2si had negligible effects on VCAM1 mRNA in vehicle- or dex-treated cells, while a slight decrease was observed in both TNFα- (0.7-fold) and TNFα+dex-treated cells (0.8-fold) relative to NTsi suggesting that DAB2 may increase VCAM1 levels in some contexts. In summary, these results suggest that DAB2 may inhibit the expression of SOD2 and CTSB in vehicle- and/or dex-treated cells, and potently inhibit SOD2 in the presence of TNFα. The effect of DAB2 on GR-induced genes has not yet been investigated.
5.4.4. **DAB2 does not alter RelA mRNA expression**

To begin to understand how DAB2 affects NFκB activity and target gene expression, we first set to determine if DAB2 impacts levels of RelA, the active subunit of NFκB and the primary target of transrepression by GR in regulation of inflammatory signalling. RT-qPCR for RelA was first performed using the same cDNA used to determine target gene levels 24h following vehicle or dex treatment in Figure 5.4A-C. No difference was observed with vehicle vs. dex treatment in cells transfected with NTsi, while dex treatment led to a small (1.2-fold) but statistically significant increase in RelA mRNA in DAB2si-transfected cells (p<0.05, Figure 5.5A). Within treatments, DAB2si had no effect in vehicle-treated cells and a 1.5-fold increase in RelA was observed in dex-treated cells, although this did not reach statistical significance.

RT-qPCR for RelA was then performed using the same cDNA used in Figure 5.4D-F harvested 8h following vehicle, dex and/or TNFα treatment. In cells transfected with NTsi, no difference in RelA was observed with TNFα or TNFα+dex treatment relative to vehicle while a slight decrease (0.8-fold) was seen with dex treatment alone (Figure 5.5B). In contrast, in DAB2si-transfected cells RelA mRNA was unchanged with dex treatment alone and increased with both TNFα (1.4-fold) and TNFα+dex (1.6-fold) treatment relative to vehicle. Overall, DAB2si slightly decreased RelA mRNA levels in vehicle-treated cells, while negligible effects were observed in cells treated with dex and/or TNFα. In summary, DAB2 does not appear to appreciably alter RelA mRNA expression. The impact of DAB2 on RelA protein levels has not yet been investigated.

5.4.5. **DAB2 interacts with GR and RelA proteins in vehicle and dex-treated cells**

To determine whether DAB2 may impact GR and NFκB activity through protein-protein interactions, immunoprecipitation for DAB2 was performed on ES2 lysates previously treated with vehicle or dex, followed by immunoblotting for GR and RelA proteins. Figure 5.6 presents preliminary data showing the presence of bands representing GR and RelA in vehicle- and dex-treated cells, suggesting that DAB2 interacts with both proteins (additional experiments are
FIGURE 5.5: DAB2 does not alter RelA mRNA expression. RT-qPCR for RelA (using the same cDNA described in Figure 5.4A-C) revealed no significant impact of DAB2si in cells treated with vehicle or dex for 24h (A) (n=3 experiments). RT-qPCR using the same cDNA described in Figure 5.4D-F revealed a slight decrease in RelA mRNA in DAB2si-transfected cells treated with vehicle for 8h, whereas negligible effects were observed in cells treated with TNFα and/or dex for the same time period (B) (n=1 experiment). RelA mRNA expression (normalized to actin) relative to NTsi-transfected, vehicle-treated cells are shown in A and B. Different letters shown in A indicate statistically significant differences in average mRNA expression (as determined by one-way ANOVA followed by Newman-Keuls Multiple Comparison Test; p < 0.05).
FIGURE 5.6: DAB2 interacts with GR and RelA proteins in vehicle- and dex-treated cells.

Immunoprecipitation (IP) was performed on ES2 lysates using anti-DAB2 antibody, followed by immunoblotting for GR and RelA. Both GR and RelA proteins were detected in cells previously treated with dex or vehicle, suggesting that endogenous DAB2 interacts with both proteins. IP was also performed using IgG as a negative control.
ongoing to verify these interactions). No such bands are observed when immunoprecipitation was performed using IgG control. Although the bands for GR and RelA appear fainter in dextratreated cells, this technique is not quantitative and may also reflect downregulation of both proteins in this context. On the whole, these results suggest that DAB2 enhances GR transactivation and transrepression activity and subsequently reduces the expression of select NFκB target genes, and may do this in part via interaction with GR and RelA proteins.

5.5. DISCUSSION

We have previously demonstrated decreased mRNA and cytoplasmic protein expression of the tumour suppressor DAB2 in SerCa and FTEb luteal samples (Chapter 2). As outlined in the previous chapter, the same groups of samples show evidence of diminished GR signalling and elevated NFκB-dependent inflammatory signalling. Intriguingly, secretory but not ciliated FTE cells showed intense staining for the active NFκB subunit RelA and conversely specific loss of cytoplasmic DAB2 staining during the luteal phase, suggesting a potential inverse relationship between these two proteins. Given previous reports of negative regulation of AR by DAB2 (298), we hypothesized that DAB2 may also be involved in regulation of an additional steroid hormone receptor, namely regulation of anti-inflammatory signalling by the GR.

In order to investigate the role of DAB2 in GR-dependent signalling, we screened both ovarian (ES2, SKOV3) and breast (MCF7, BT20) cancer cell lines for high transfection efficiency, glucocorticoid-responsiveness and endogenous expression of our proteins of interest (GR, RelA, DAB2). Only ES2 ovarian cancer cells fulfilled all of these criteria, therefore all experiments in this study were performed using this model. In the future similar experiments should also be done using primary (or immortalized) cultures derived from FTE cells; unfortunately our attempts to optimize the use of these cells for this purpose have thus far been unsuccessful. Importantly, in ES2 cells both RT-qPCR and Western blotting confirmed the
downregulation of GR in response to dex treatment, further supporting our hypothesis that the increased GR mRNA observed in FTEb(S) and SerCa samples in the previous chapter are reflective of decreased or disrupted GR signalling.

In light of our earlier finding that a subset of GR-induced genes show decreased expression in DAB2-deficient FTEb(S) samples, we first set to determine whether DAB2 directly increases GR transactivation activity. Indeed, transfection of ES2 cells with a DAB2 expression construct resulted in a 2.5-fold enhanced induction of (GRE-containing) MMTV luciferase activity with dex treatment relative to empty vector. This suggests that DAB2 may be functioning as a co-activator for GR-dependent transcription similar to known co-activators CBP, SRC1 and GRIP1 (158, 254). Interestingly, one study reported the presence of a GRE in the mouse DAB2 promoter (309), suggesting that induction of DAB2 by glucocorticoids may represent a potential feed-forward mechanism.

Previous studies have implicated DAB2 in the facilitation of TGFβ-induced growth inhibition (214) and the propagation of the canonical and non-canonical TGFβ signalling pathways. In the canonical pathway, DAB2 facilitates the phosphorylation of Smad proteins by the activated TGFβ receptor complex, translocation of activated Smad proteins to the nucleus and subsequent activation of Smad-dependent transcription (214, 303). This was found to occur via interaction of DAB2 with both the type I/II TGFβ receptors and their phosphorylation target proteins Smad2 and Smad3 (214). DAB2 has also been found to stimulate TGFβ-induced JNK activation, fibronectin expression and cell migration independent of Smad proteins, via association of DAB2 with (the mitogen-activated protein kinase kinase kinase) TAK1 (303). A separate study observed accumulation of DAB2 at the cellular membrane upon TGFβ stimulation of mouse mammary gland epithelial cells, where it associated with and activated β1-integrin via focal adhesion kinase phosphorylation (300). This led to epithelial-to-mesenchymal transition, suggesting DAB2 may have a dual role in tumourigenesis similar to TGFβ itself. It is noteworthy that in the activation of both the canonical and noncanonical
pathways, DAB2 activates TGFβ signalling components by bringing together an activating kinase and its target protein.

A more striking finding in the previous chapter was that DAB2-deficient FTEb(S) samples showed increased expression of a large proportion of GR-repressed genes, most notably those implicated in inflammatory signalling, and additional NFκB-induced genes. We therefore performed a preliminary experiment to determine whether DAB2 may also enhance GR transrepression of NFκB activity. Accordingly, transfection with DAB2si enhanced NFκB-responsive luciferase activity relative to NTsi within all treatment groups (vehicle, dex, TNFα, TNFα+dex), with a 2.2-fold induction observed in cells treated with the NFκB ligand TNFα. Overall, cells transfected with DAB2si showed a 1.4-fold enhanced TNFα-induced NFκB activity relative to NTsi, a difference that may be minimized by the increased basal NFκB activity in DAB2si-transfected cells. Importantly, dex treatment decreased TNFα-induced but not basal NFκB activity in all transfected cells, highlighting the involvement of GR in inhibition of NFκB in our model system. These preliminary findings suggest that DAB2 may function as a co-repressor for NFκB-dependent transcription similar to GR.

To determine if the effect of DAB2 on GR and NFκB reporter activity resulted in altered gene transcription, RT-qPCR was performed for the NFκB target genes SOD2, CTSB and VCAM1 following transfection with DAB2si. In the previous chapter, FTEb(S) and SerCa samples showed altered expression of all 5 probe sets corresponding to SOD2, with an average 10.9-fold increase relative to FTEb(N) samples. Consistent with the observed effect on luciferase activity, transfection of ES2 cells with DAB2si increased SOD2 mRNA levels relative to NTsi in dex-, TNFα- and TNFα+dex-treated cells. The greatest effect was observed following TNFα treatment, with cells transfected with DAB2si showing an 8.6-fold increase in SOD2 relative to vehicle compared to a 4.2-fold increase observed in NTsi-transfected cells. Similar to SOD2, FTEb(S) and SerCa samples showed elevated expression of 5 probe sets corresponding to CTSB in the previous chapter (average of 2.4-fold increase) and 1 probe set corresponding to VCAM1 (15.7-fold). Unlike SOD2, transfection of DAB2si in the present study
only slightly increased CTSB mRNA expression in vehicle- and dex-treated cells at 24h and TNFα- and TNFα+dex-treated cells at 8h. CTSB was also not induced by TNFα treatment in general, suggesting that it may not be a target of TNFα-dependent signalling in our model system. Finally, DAB2si did not alter VCAM1 mRNA levels in vehicle- and dex-treated cells, while it slightly decreased VCAM1 in TNFα- and TNFα+dex-treated cells. This suggests that DAB2 may actually induce VCAM1 levels in some contexts, although this needs to be confirmed by further experiments. In summary, these results suggest that DAB2 strongly inhibits mRNA expression of SOD2, with minimal effects on CTSB or VCAM1 levels despite a considerable impact on NFκB luciferase activity. Experiments will therefore be performed to identify additional NFκB-induced genes, as well as primary GR targets, influenced by DAB2. Emphasis will be placed on those genes previously found to be altered in DAB2-deficient FTE and SerCa samples. Western blot analysis will also be performed to verify the current findings at the protein level.

Previous studies have identified several mechanisms of regulation of diverse signalling pathways by DAB2. These mechanisms are not mutually exclusive and have included alteration of the phosphorylation status of key molecules, protein-protein interactions resulting in altered recruitment/access to targeting phosphatases/kinases or disruption of protein complexes, and altered subunit expression. For example, DAB2 inhibits AR-mediated cell growth by interaction with c-Src protein, disrupting binding of c-Src to AR and dramatically reducing AR-induced Src phosphorylation (298, 306). Similarly, DAB2 inhibits the canonical Wnt signalling pathway by interacting with both disheveled-3 and axin, disrupting their association (297). DAB2 has also been found to inhibit AP-1 activity in many studies. The AP-1 transcriptional complex, composed of subunits c-Fos and c-Jun, is involved in key processes such as cell proliferation, differentiation and malignant transformation (149, 304). Similar to NFκB, a subset of AP-1 target genes have been implicated in inflammatory signalling, and AP-1 is a target of negative regulation by GR (149). Negative regulation of both NFκB and AP-1 activity by DAB2 would therefore position DAB2 as an important anti-inflammatory mediator. In
the first study, DAB2 was found to inhibit TPA-induced AP-1 activity in prostate epithelial cells; this required protein kinase C (PKC)-dependent phosphorylation of the N terminus of DAB2 at Ser24 (305). Detailed sequence analysis surrounding the phosphorylation site revealed a potential nuclear localization signal, providing a potential explanation for the nuclear inhibition of AP-1 by DAB2. A separate study similarly demonstrated decreased expression of the AP-1 subunit c-Fos in DAB2-expressing cells, via reduced serum-stimulated phosphorylation of the activating transcription factor Elk-1 (304). While DAB2 was not found to directly interact with Elk-1, the authors proposed that DAB2 may either recruit serum/threonine-protein phosphatase 2B (PP2B) or directly inhibit MAPK-dependent phosphorylation of Elk-1 by preventing MAPK nuclear entry or access of the activated kinase to its target. Finally, DAB2 can also inhibit growth factor-induced AP-1 activity by binding of its C-terminal (proline-rich) domain to the SH3 domain of the adaptor molecule growth factor receptor binding protein 2 (Grb2), disrupting the interaction between Grb2 and the guanine nucleotide exchange factor son of sevenless (298, 306, 310).

To begin to understand how DAB2 affects NFκB activity and target gene expression, we first set to determine if DAB2 impacts levels of RelA, the active subunit of NFκB and the primary target of transrepression by GR in regulation of inflammatory signalling. RT-qPCR revealed that DAB2si had negligible effects on RelA mRNA in cells treated with dex and/or TNFα for 8h, while a modest yet statistically insignificant increase was observed in cells treated with dex for 24h. Therefore DAB2 does not appear to appreciably alter RelA mRNA expression, suggesting that DAB2 impacts the GR-RelA pathway through a different mechanism. It will be important to extend these observations to the protein level and these experiments are underway. It is also unclear at this time whether the increased expression of RelA observed in FTEb(S) samples is directly related to the decreased DAB2 observed in the same samples, or whether this is a consequence of the inflammatory environment following ovulation. Immunoprecipitation for DAB2 followed by immunoblotting for GR and RelA revealed that DAB2 interacts with both proteins in vehicle- and dex-treated ES2 cells. Although this
experiment should also be performed in cells treated with TNFα, these results indicate protein-protein interactions may be a mechanism by which DAB2 enhances GR transactivation and transrepression activity.

In summary, overexpression of the tumour suppressor DAB2 enhances dex-induced GR transactivation activity in ES2 ovarian cancer cells, indicating a novel function for DAB2 as a modulator of GR signalling. In addition, siRNA-mediated knockdown of DAB2 potently increased TNFα-induced NFκB activity and SOD2 mRNA expression, providing strong evidence for DAB2-enhanced GR-mediated transrepression of NFκB. Endogenous DAB2 protein was found to interact with both GR and RelA, providing a potential mechanism for these effects. Altogether, these findings support a novel pathway whereby DAB2 influences the activity of GR and NFκB proteins and subsequent inflammatory gene expression.
CHAPTER 6
GENERAL DISCUSSION

In light of recent evidence strongly implicating the FTE as the source of ovarian high-grade SerCa, the studies outlined in this thesis were directed at identifying molecular alterations in non-malignant FTE from BRCA mutation carriers that may predispose to malignant transformation. Discovery and characterization of these molecular alterations could contribute to a better understanding of the earliest events of serous carcinogenesis, which could lead to improved detection of those patients at greatly enhanced risk, prevention, and ultimately significant reductions in ovarian cancer-associated mortality.

In work described in Chapter 2, gene expression profiles of microdissected epithelial cells from FTEa, FTEb and SerCa specimens were generated and compared. Importantly, both cluster and significance analysis indicated that SerCa have similar molecular profiles whether of presumed ovarian or tubal origin, providing further support that they share a common cell of origin within the FTE. Furthermore, gene expression profiles of FTE specimens varied according to the stage of the ovarian cycle, with BRCA1/2 mutation carriers showing an increased number of differentially expressed probe sets between the follicular and luteal phases compared to normal controls. This finding is consistent with the role of BRCA1/2 proteins in modulating steroid hormone receptor (AR, PR and ER) activity (Section 1.4.4), and provides further support for the hypothesis that ovarian hormones are relevant to SerCa (Sections 1.9-1.10). In addition, FTEb as a group, and four individual FTEb samples from the luteal phase in particular (referred to throughout as FTEb(S)), showed gene expression profiles that closely resembled those of SerCa samples. Intriguingly, three of the four FTEb(S) samples were obtained from carriers of the BRCA1 185delAG mutation. Also, of the 6 samples with this mutation included in this study, zero of three obtained during the follicular phase (vs. three of three from the luteal phase) clustered with SerCa samples. Further study of a large number of FTE samples comparing the implications of BRCA1 185delAG and other specific mutations...
could therefore be of value. Altogether, the findings presented in this chapter suggest that FTE from \textit{BRCA1/2} mutation carriers exhibit gene expression changes that parallel those observed in (both tubal and ovarian) SerCa, and further that factor/s associated with the luteal phase direct these changes.

At this time, it is important to note two limitations of this study that emerge in retrospect. Firstly, given the high frequency of p53 signatures in FTE which was reported after my study (180, 197, 198), it is possible that p53-mutated cells were inadvertently captured during the LCM process and could impact gene expression profiling. As outlined in Section 1.12.4, p53 signatures occur in benign-appearing epithelium, and IHC for p53 was not performed prior to LCM. However, these lesions have been reported to occur with similar frequency in the FTE of mutation carriers and controls, therefore the chance of including these cells in either group would likely be equivalent.

Secondly, although my findings strongly suggest that the milieu of the luteal phase is an important determinant of gene expression in FTE cells, the unique hormone profile of each patient was not examined. I had initially stratified samples by the phase of the menstrual cycle to circumvent variability introduced by the dramatically different follicular and luteal phase hormone profiles, which could target the FTE. My finding that gene profiles generated from luteal phase FTEb samples best resembled SerCa was initially surprising and seemed counterintuitive given the evidence for a protective role of progesterone in ovarian carcinogenesis (Section 1.9.1). Through careful review of ovarian and uterine slides we were able to determine the phase of the cycle (luteal vs. follicular); however, corresponding blood, urine or follicular fluid samples were not analyzed. It is therefore possible that some of the included patients (most notably those women from which FTEb(S) samples were obtained) have (unknown) altered levels in a specific hormone or group of hormones, perhaps consistent with a previous report suggesting that mutation carriers initiate menopause at an earlier age than normal controls (213). A more thorough characterization of the hormonal and other parameters of the luteal phase should therefore be incorporated in future studies.
It is also important to point out additional technical limitations that apply to this thesis as a whole. Firstly, due to the use of limited human tissues, a relatively small number of samples were subjected to gene expression profiling. Secondly, no in-house studies on the potential effects of linear amplification of mRNA were performed, largely because this methodology has been extensively validated by the staff at NuGEN (203, 311). Despite these concerns, extensive measures have been taken to limit any technical sources of variation. For instance, I personally completed LCM and RNA extractions for all samples, and one person (Monika Sharma from the UHN Microarray Center) was responsible for all amplifications and hybridizations. Also, all samples were subjected to linear amplification (even SerCa specimens with sufficient RNA for profiling) to ensure equal bias, and all NuGEN kits and GeneChip arrays were obtained from the same batch. An additional point is that all IHC was performed as a service provided by the UHN Pathology Research Program Lab. As part of their work-up for each antibody, various dilutions are performed on control sections, including the use of non-specific IgGs. The use of competing peptide antigens to further validate the antibodies was not performed. Finally, only subjective semi-quantitative IHC analysis was performed. The results are currently being validated through the use of image analysis quantifying the percent of cells stained per field and the intensity of the staining product.

An initial hypothesis for the clustering of FTEb with SerCa was that FTEb may respond differently to progesterone than FTEn, and that this altered response contributes to an increased propensity for malignant transformation. As previously discussed, progesterone is specifically elevated during the luteal phase, and although generally considered a protective factor, recent studies suggest that luteal-phase levels of progesterone may have growth-promoting effects (109, 240). I therefore determined the expression of PR in FTE and SerCa specimens in studies described in Chapter 3, using RT-qPCR and isoform-specific IHC. SerCa specimens exhibited decreased nuclear PRA and PRB protein compared to normal controls, whereas preferential loss of nuclear and cytoplasmic PRA protein was observed in luteal relative to follicular FTE samples. The resulting PRB predominance in luteal samples (obtained
from both mutation carriers and controls) may contribute to the differential gene expression profiles observed during this phase, and may therefore be of interest to investigators in the field of reproductive biology since secreted tubal factors are thought to support secondary oocytes and preimplantation embryo development (166, 168). For the purpose of this thesis, however, the finding of similar relative isoform expression in FTEn and FTEb samples (as well as FTEb(S) and FTEb(N) samples) suggests that differential response to progesterone is not likely the determining contributor to predisposition to SerCa. To complement the IHC approach used in this study, quantification of PRA/PRB levels by Western blotting of (micro- or macrodissected) snap-frozen tissue from these and additional samples could be performed to further demonstrate that relative isoform levels are not altered according to BRCA mutation status. Expression of PR co-activators and co-repressors could also be investigated, as it remains possible that unidentified changes in these proteins could alter the response of select FTE to luteal progesterone.

Despite this possibility, the studies described in Chapters 2 and 3 collectively indicate that altered response to or presence of a factor associated with the luteal phase contributes to (potentially predisposing) gene expression changes in FTEb specimens, and further that this factor is likely not progesterone. As previously discussed (Section 1.8.2), the luteal phase is characterized by an elevation of inflammatory cytokines (e.g. TNFα, IL-1) at the ovarian surface (and presumably adjacent FTE), leading to activation of pro-inflammatory signalling by NFκB and compensatory anti-inflammatory signalling by GR. I therefore investigated the hypothesis that FTEb/FTEb(S) samples have altered GR and/or NFκB signalling during the luteal phase in studies described in Chapter 4, by characterizing the expression of GR, RelA and their respective targets in FTE and SerCa specimens. Overall, FTEb(S) samples showed evidence of diminished GR signalling relative to FTEb(N), characterized by an elevation of GR mRNA/nuclear protein, and altered expression of both GR-induced and GR-repressed (especially pro-inflammatory) genes. FTEb(S) samples also showed evidence of elevated NFκB-dependent signalling, characterized by increased expression of RelA and a large
proportion of NFκB-induced genes. Most notably, many differentially expressed genes were similarly altered in SerCa, suggesting that altered expression of GR- and NFκB-influenced genes contributes to the similarity of FTEb(S) and SerCa specimens. To my knowledge, this study is the first to suggest a protective role for GR signalling against SerCa development, and could open up an exciting new area of study in ovarian cancer biology.

An important question that remains is whether FTEb(S) samples show altered inflammatory gene expression profiles as a result of prophylactic surgery occurring closer to the time of ovulation (an inflammatory event) compared to other luteal samples. This does not appear to be the case, as endometrial dating of a sampling of profiled cases has thus far revealed no difference in the timing of surgery relative to ovulation in FTEb(S) (or the 3 FTEn luteal samples clustering with FTEb(S) according to NFκB target gene expression) compared to other luteal samples. In fact, the two FTEb(S) samples examined thus far were obtained at cycle days 24 and 23 (sample # 14 and 15 respectively), suggesting that increased pro-inflammatory signalling has continued for many days following ovulation at day 14. Although the normal timing of inflammatory signalling within human FTE following ovulation is presently unknown, recent study of primary monocytes suggests that cytoplasmic GR transrepression activity is activated within hours of glucocorticoid exposure, resulting in rapid cessation of pro-inflammatory gene induction (312).

The finding of altered inflammatory signalling in FTE of women at increased risk for SerCa is consistent with studies implicating inflammation as a risk factor for ovarian cancer, most notably epidemiologic studies showing a protective effect of NSAIDs. It also provides justification for further investigation into the use of NSAIDs for ovarian cancer prevention in high-risk women, similar to the use of the COX2 inhibitor celecoxib for the prevention of colon cancer in patients with familial adenomatous polyposis (313). In the ovarian cancer field, investigators have historically attributed the protective effect of NSAIDs to inhibition of inflammatory signalling and malignant transformation of OSE cells. However, to my knowledge a definitive study that directly addresses this hypothesis has not been performed. It therefore
remains a strong possibility that NSAIDs could instead be functioning through prevention of malignant transformation of FTE cells. To address this alternative hypothesis, more detailed epidemiologic studies on the association of use of NSAIDs with p53 signatures and TICs/early tubal cancers in prophylactic samples from both BRCA mutation carriers and control patients should be performed. The association of NSAIDs with alterations in OSE morphology should be included as a comparison. The direct effect of NSAIDs on primary FTE cell cultures should also be investigated, specifically the impact on growth properties and gene expression changes described in this study.

Collectively, the studies presented in Chapters 2-4 suggest that altered inflammatory, rather than progesterone, signalling in FTE cells during the luteal phase is an important contributor to predisposition to SerCa. I therefore began to explore potential mechanisms by which this altered response could occur in studies described in Chapter 5, using a glucocorticoid- and cytokine-responsive cell model (ES2 ovarian cancer cells). Specifically, I hypothesized that DAB2, an adaptor protein found to be decreased in SerCa and FTEb luteal samples as presented in Chapter 2, may activate GR signalling, including GR-dependent repression of RelA. This hypothesis was based on two main observations, namely the previously reported inhibition of AR by DAB2 and the inverse pattern of immunopositivity of DAB2 and RelA exhibited by secretory FTE cells during the luteal phase. Altogether, the experiments in ES2 cells demonstrate that DAB2 promotes both GR-mediated transactivation and suppression of NFκB signalling (including expression of the NFκB target SOD2), in part via interaction with GR and RelA proteins. While confirmatory experiments in primary FTE cells are imperative, these findings, combined with those reported in Chapter 2, suggest that DAB2 is a crucial determinant of inflammatory signalling and ovarian cancer risk. Further characterization of how the interaction of DAB2 with GR and RelA proteins impacts GR- and NFκB-dependent transactivation and transrepression in future studies may therefore greatly impact our understanding of early events of SerCa.
One possibility that will be investigated is that DAB2 may alter the phosphorylation status of GR or RelA, potentially through altered recruitment or access to a specific targeting phosphatase or kinase (similar to its regulation of the AR and Wnt signalling pathways). For instance, DAB2 may associate with and/or affect the interaction of GR and RelA proteins with glycogen synthase kinase 3 beta (GSK3β), a kinase previously shown to have opposing effects on GR and RelA function. While hormone-dependent phosphorylation of GR has been shown to inhibit receptor-mediated repression of NFκB transcription (251), phosphorylation of RelA by GSK3β directly enhances its transactivation activity (152). Alternatively, DAB2 may impact the recruitment of and/or access to the critical regulator PKA to cytoplasmic GR and RelA proteins and subsequently alter the complex dynamics of PKA-dependent cross-repression. It has previously been reported that PKAc interacts with and phosphorylates cytoplasmic RelA at Ser276 upon ligand stimulation and degradation of inhibitory IκB proteins (150, 152, 153). Phosphorylation at this site enhances the ability of RelA to recruit co-activator proteins such as CBP (152) and RelA-dependent transcription (150). Inhibition of GR-dependent transactivation has also been found to be dependent on cytoplasmic PKA-phosphorylated RelA. At the same time, GR-mediated inhibition of NFκB-dependent transcription is dependent on PKAc. Specifically, GR competes with RelA for binding to PKAc, resulting in repression of PKAc-dependent NFκB transcription. Finally, overexpression of PKAc both enhances GR transactivation activity (as measured by MMTV luciferase induction) and rescues GR from inhibition by RelA (150).

In addition to identifying potential co-factors of DAB2, current studies are directed at determining the subcellular localization of these interactions. Preliminary immunofluorescence experiments suggest that DAB2 protein is present in both the cytoplasm and nucleus similar to GR and RelA. It is therefore conceivable that DAB2 may also be present at regulatory sites (GRE or κB sites) in promoters of altered target genes such as SOD2. Future studies could also be performed to determine potential mechanisms of DAB2 downregulation in FTE cells, such as promoter hypermethylation or single nucleotide polymorphisms.
6.1. An alternative model for serous carcinogenesis

Although many potential explanations for malignant transformation of OSE have been put forth – most notably the Incessant Ovulation hypothesis, the Inflammation hypothesis and the hormonal hypotheses – a unifying theory that can account for the majority of clinical/pathological, molecular and epidemiological observations has thus far not been proposed. It is also important to note that many of these hypotheses (as well as the epidemiological and molecular studies on which they are based), do not differentiate by histological type, despite overwhelming evidence that the histological subtypes signify distinct diseases. Recent evidence implicating the FTE as the source of high-grade SerCa, combined with the data presented in this thesis, supports the generation of an alternative model for serous carcinogenesis which builds on that recently articulated by Crum et al (17) and incorporates elements of each of the earlier hypotheses. This model proposes that malignant transformation of the secretory epithelial cells of the distal fallopian tube (e.g. progression from normal FTE → p53 signatures → TICs → invasive SerCa) is promoted by an altered balance of pro- vs. anti-inflammatory signalling during the post-ovulatory luteal phase, and further that this balance is determined by the underlying status of key genes (e.g. DAB2 and BRCA1). In cells proficient for DAB2, GR is activated shortly following ovulation (through cytokine-dependent induction of cortisol formation and co-activator function of DAB2), leading to cessation of NFκB-dependent pro-inflammatory signalling (Figure 6.1A). In cells deficient for DAB2, anti-inflammatory signalling by GR is not activated following ovulation, shifting the balance in favour of pro-inflammatory signalling (Figure 6.1B). Incessant activation of the inflammatory response by NFκB during successive luteal phases in DAB2-deficient cells could eventually lead to genomic instability and increased tumour risk, through continued production of mutagenic reactive oxygen species and induction of cell cycle and anti-apoptotic genes. Preliminary experiments (Tone, unpublished) showing enhancement of GR transactivation activity by BRCA1 (by an as yet unidentified mechanism) also suggest that BRCA1 may be involved in
A. FTE luteal cells with proficient DAB2:

- Glucocorticoids
- Pro-inflammatory cytokines (IL-1, TNFα)

Cytoplasm

- Hsp90
- P
- GR
- DAB2
- CBP
- mRNA
- GRE
- Anti-inflammatory
- mRNA
- Pro-Inflammatory

Nucleus

- RelA
- p50
- IκB
- PKA

B. FTE luteal cells with deficient DAB2:

- Glucocorticoids
- Pro-inflammatory cytokines (IL-1, TNFα)

Cytoplasm

- Hsp90
- P
- GR
- DAB2
- CBP
- mRNA
- GRE
- Anti-inflammatory
- mRNA
- Pro-Inflammatory
FIGURE 6.1: Proposed model for serous carcinogenesis incorporating a central role for DAB2. In this model, the risk of malignant transformation of distal (secretory) FTE is increased by an altered balance of pro- vs. anti-inflammatory signalling during the post-ovulatory luteal phase, as determined by DAB2 status. In cells with sufficient DAB2 (A), DAB2 enhances GR transactivation activity (resulting in increased synthesis of anti-inflammatory genes) shortly following ovulation, potentially through binding of GR at GRE sites within the promoter region of anti-inflammatory genes (1). DAB2 also enhances GR transrepression activity (resulting in decreased synthesis of pro-inflammatory genes), potentially through binding of GR and RelA proteins (and potentially regulatory kinases such as PKA) in the cytoplasm (2), or alternatively at NFκB sites within the promoter region of pro-inflammatory genes (3). Altogether, these actions of DAB2 would shift the balance in favour of anti-inflammatory signalling, leading to resolution of the pro-inflammatory milieu which promotes mutagenesis. In contrast, cells with deficient DAB2 (B) would have diminished GR signalling following ovulation, slowing the resolution of the pro-inflammatory environment by prolonging NFκB-dependent pro-inflammatory signalling. Thus the model is consistent with a role for incessant ovulation, but places an emphasis on the ability to maintain homeostasis within the distal tube compartment.
this novel pathway.

Similar to the Incessant Ovulation hypothesis, this revised model is consistent with findings that risk of ovarian cancer increases with repeated ovulations. An important contrast, however, is that my proposed model emphasizes the impact of the unique hormonal/inflammatory environment associated with ovulation rather than proliferative repair of epithelial cells. The current model also differs from both the Incessant Ovulation and Inflammation hypotheses in the proposed target cell type (FTE vs. OSE). Although others have proposed a role for tubal inflammation in carcinogenesis, these studies have largely focused on retrograde menstruation rather than ovulation-associated inflammatory signalling (78). Finally, the current model proposes a central protective role for glucocorticoids/GR signalling in ovarian cancer, in contrast to previous hormonal hypotheses which focus on ovarian steroids (estrogens, progesterone or androgens).

The proposed model is also consistent with key epidemiologic observations, most notably the association of risk with the number of lifetime ovulations and use of NSAIDs. While the protective effect of tubal ligation is consistent with a tubal origin of SerCa, the current model may also provide an explanation for why the associated odds ratio is not zero. For instance, following tubal sterilization procedures other than fimbriectomy, the distal FTE would presumably remain exposed to ovulation-associated inflammatory mediators at the ovarian surface and hence remain at an increased risk for malignant transformation. As epidemiologic studies on tubal ligation to date do not indicate or differentiate between the specific procedures used, it is possible that inclusion of all methods could confound the results. A definitive study for the association of specific procedures of tubal ligation on SerCa risk should therefore be performed.

To begin to test the proposed model, FTE could be microdissected from the distal fallopian tube both ipsilateral (FTE_{ips}) and contralateral (FTE_{con}) to the ovulating ovary, followed by determination of DAB2 (and BRCA1/2) status and gene/protein expression profiling. IHC for the secretory cell marker HMFG2 could be performed prior to LCM to enrich for this cell type. In
addition, samples could be included from multiple specified times following ovulation, as determined by endometrial dating. Corresponding blood and follicular fluid samples should also be collected and analyzed at each time point for various hormones and factors associated with the luteal phase, including but not limited to progesterone, cortisol and inflammatory cytokines (e.g. TNFα and IL-1). While progesterone levels would likely be similar both proximal and distal to the ovulatory site, cortisol and inflammatory cytokines would be elevated proximal to the ovulating ovary based on previous studies (Section 1.8.2). Comparison of profiles of FTE_ips and FTE_con samples from the same individual and as a group would reveal the impact of the local inflammatory environment on gene and protein expression. Given the crucial role of NFκB in response to inflammatory cytokines, FTE_ips samples would be expected to exhibit increased expression of several NFκB target genes such as those highlighted in this thesis. I predict that the differences between FTE_ips and FTE_con would be altered and/or amplified in those patients with deficient DAB2, that DAB2-deficient samples would show a shift towards pro-inflammatory gene expression, and that these changes would parallel those found in SerCa specimens. Furthermore, the duration of pro-inflammatory signalling may be extended in DAB2-deficient samples that are unable to activate compensatory anti-inflammatory signalling by GR.

If DAB2-deficient FTE showed evidence of increased or extended pro-inflammatory signalling as hypothesized, the direct impact on tubal carcinogenesis in vivo could be tested in an animal model engineered to have a DAB2-deficient or wild-type background. Unfortunately no mouse model of fallopian tube SerCa currently exists. There are also important limitations to consider if a mouse model is developed/used to test this hypothesis, including the fact that the mouse fallopian tube (oviduct) is partly contained within the ovarian bursa and that mice do not experience a (luteal) rise in progesterone unless the cervix is stimulated (such as occurs with mating) (314). To my knowledge, it is unknown whether post-ovulatory glucocorticoid/GR and inflammatory signalling is comparable in mice and humans.

In conclusion, this thesis identifies gene expression changes in non-malignant FTE from BRCA mutation carriers during the post-ovulatory luteal phase that parallel those detected in
high-grade SerCa. The data support the generation of a novel testable model for predisposing events contributing to high-grade serous carcinogenesis that centres on an altered ability to quickly resolve the pro-inflammatory environment created by the ovulatory event. This model could be extended to include additional pro-inflammatory events that may impact the distal FTE, including retrograde menstruation and PID (Figure 6.2). As such, this model provides an important framework upon which to base further studies. The work presented in this thesis thus increases our understanding of early predisposing events associated with ovarian carcinogenesis, providing a better platform for discovery of meaningful markers of early disease/increased risk, and offers directions for development of prevention strategies.
FIGURE 6.2: Risk of SerCa is determined by the balance of pro- and anti-inflammatory signalling. In this model, serous carcinogenesis is promoted by a diminished ability of GR pathway-deficient distal FTE cells to restore homeostasis following inflammatory events in general, including ovulation, retrograde menstruation and PID.
CHAPTER 7
REFERENCES

183


314. Brown, T. J. Personal communication.