THE ROLE OF HUMAN PYGOPUS 2 IN THE PROLIFERATION OF OVARIAN CANCER CELLS

MALCOLM WELLS
The role of Human Pygopus 2 in the proliferation of ovarian cancer cells

By

Malcolm Wells, B.Sc.(Hons) MD

A thesis submitted to the School of Graduate Studies
In partial fulfillment of the requirements for the Degree of Master of Science

Faculty of Medicine
Memorial University of Newfoundland
May, 2009

St. John’s

Newfoundland and Labrador
Pygopus plays an important role in canonical Wnt signaling by functioning in complex with T cell factor (TCF), B Cell Lymphoma 9 (BCL9) and β-catenin to activate target gene transcription. Many cancers show mutational defects in components of the Wnt pathway, leading to β-catenin overexpression and increased TCF-mediated transcription of the target genes. Pygopus is crucial to this target gene overexpression in that it is a required protein in the transcription complex. As such, we show pygopus is overexpressed in Epithelial Ovarian Cancer (EOC) cell lines and is required for their proliferation. To date pygopus function has only been studied in relation to its canonical Wnt signaling activity. However, we also demonstrate that pygopus functions outside the canonical Wnt pathway as a transactivator and that its presence is required for EOC proliferation regardless of whether it functions in the canonical Wnt signaling pathway.
ACKNOWLEDGEMENTS

First, I would like to thank my supervisors Dr. Ken Kao and Dr. Cathy Popadiuk, for the opportunity to study and work as a graduate student in their laboratory. Obtaining my M.Sc. means a lot to me and I thank them for their support in this pursuit.

I would also like to thank my committee members, Dr. Gillespie and Dr. Paradis, for their helpful comments and suggestions, both during my committee meetings and with the review of this thesis.

A big thank you goes out to my coworkers. Jieying Xiong, Blue Lake, Rebecca Ford, Kelly Downton, Phil Andrews, Mark Kennedy, and everyone else in the Terry Fox Cancer Research Laboratories were always a source of friendship and advice.

On a personal note, I would like to thank my girlfriend, S. Jane Buffett, for her support during medical school and the writing of this thesis. I also send a big thank you to my parents Scott and Amanda, my brother Michael, my grandparents and family for their love and support throughout this entire process.
## TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>i</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>ii</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>iii</td>
</tr>
<tr>
<td>List of Tables</td>
<td>v</td>
</tr>
<tr>
<td>List of Figures</td>
<td>vi</td>
</tr>
<tr>
<td>List of Abbreviations</td>
<td>vii</td>
</tr>
</tbody>
</table>

### Chapter 1: Introduction

1.1 Ovarian Cancer
   1.1.1 Incidence and Epidemiology                                   1
   1.1.2 Histopathology                                                2
   1.1.3 Pathogenesis                                                  7
   1.1.4 Risk and Protective Factors
      1.1.4.1 Race and Ethnic Factors                                  9
      1.1.4.2 Reproductive and hormonal factors                        10
      1.1.4.3 Social and Environment factors                           17
      1.1.4.4 Genetic factors
         1.1.4.4.1 Site specific ovarian cancer                         21
         1.1.4.4.2 Breast Ovarian Cancer Syndrome                       21
         1.1.4.4.3 Hereditary Nonpolyposis Colorectal Cancer            24
   1.1.5 Clinical manifestations                                       25
   1.1.6 Physical Exam                                                  27
   1.1.7 Laboratory Evaluation                                         27
   1.1.8 Diagnostic Tests                                               31
   1.1.9 Management
      1.1.9.1 Surgical Management                                      33
      1.1.9.2 Adjuvant chemotherapy                                    34
      1.1.9.3 Neoadjuvant chemotherapy                                 38
   1.1.10 Prognosis                                                    38

1.2 The Wnt Signaling Pathway                                          41
   1.2.1 Canonical Wnt Signaling                                       42
   1.2.2 Noncanonical Wnt Signaling                                    45
   1.2.3 The Role of Pygopus inCanonical Wnt Signaling                 46
   1.2.4 Wnt signaling and Disease                                     53
      1.2.4.1 Gastrointestinal Disease                                  53
      1.2.4.2 Urinary Tract Disease                                    54
      1.2.4.3 Hepatobiliary Disease                                    56
      1.2.4.4 Cardiovascular Disease                                   56
1.2.4.5 Neurologic and Psychiatric Disease ........................................ 61
1.2.4.6 Musculoskeletal Disease ...................................................... 63
1.2.4.7 Genital and Reproductive anomalies ................................. 67
1.2.4.8 Dermatologic Disease .......................................................... 70
1.3 Thesis Rationale ........................................................................... 71

Chapter 2: Materials and Methods

2.1 Cell Culture .................................................................................. 73
2.2 TCF-dependent Transcription Reporter Assays .............................. 74
2.3 Plasmids ......................................................................................... 74
2.4 Gal4-fusion Transcription Assays .................................................... 77
2.5 Protein Extraction and Western Immunoblots ................................. 77
2.6 Co-immunoprecipitations ............................................................... 82
2.7 Double Labeling Immunocytochemistry (ICC) ............................... 82
2.8 Antisense Knockdowns .................................................................. 83

Chapter 3: Results

3.1 Pygopus localizes to the nucleus in EOC cell lines ......................... 87
3.2 Endogenous Wnt activity is consistent with the presence of active
β-catenin ............................................................................................ 87
3.3 Pygopus transactivation activity is independent of β-catenin expression .... 92
3.4 Pygopus expression is required for the proliferation of EOC ............. 96
3.5 Localization of hPygo2 in dividing TOV-112D cells ......................... 101

Chapter 4: Discussion

4.1 hPygo2 transactivation activity ...................................................... 105
4.2 Wnt and cancer .............................................................................. 106
4.3 Wnt-independent functions of pygopus ......................................... 107
4.4 Pygopus as a novel therapeutic target ............................................ 109
4.5 Conclusions ................................................................................ 113
4.6 Future Directions ........................................................................ 114

Chapter 5: References ..................................................................... 117
LIST OF TABLES

Table 1.1  World Health Organization histological classification of ovarian tumors: surface epithelial-stromal tumors .......................................................... 3

Table 1.2  Ovarian Cancer Risk with respect to Contraception use and Infertility (Tworoger et al, 2007) .................................................................................. 13

Table 1.3  Summary of Selected Screening Tools for Ovarian Cancer (Nossov et al 2008) ........................................................................................................ 28

Table 1.4  Survival by FIGO stage for patient with ovarian cancer, 1996-98 FIGO statistics ........................................................................................................ 39

Table 1.5  Wnt- and β-catenin-pathway genes that are involved in diseases and syndromes (adapted from Moon et al, 2004 and http://www.stanford.edu/~musse/diseases/Humangeneticdis.htm, accessed December 21, 2008) .................................................................................. 51

Table 1.6  Comparison of phenotypes in carriers and noncarriers of LRP_R61IC (Mani et al, 2007) ................................................................................................. 57

Table 2.1  Primer Sequences and PCR Conditions for Gal-4-hPygo2 Constructs .... 80
# LIST OF FIGURES

| Figure 1.1 | Stage distribution of various types of Epithelial Ovarian Cancers (Kaku et al, 2003) | 5 |
| Figure 1.2 | Risk of Invasive Epithelial Ovarian Cancer in US White Women (Whittemore, 1994; Whittemore et al, 1992) | 11 |
| Figure 1.3 | Wnt signaling pathways (http://www.ambion.com/tools/pathway/loadImage.php?pos=bl&im=images/WNT%20Signaling.jpg) | 43 |
| Figure 1.4 | Wnt signaling pathways (Montcouquiol et al, 2006) | 47 |
| Figure 1.5 | The Wnt transcription complex (A) and 3D representation of human pygopus 2 PHD (B) | 49 |
| Figure 2.1 | Topflash Mechanism | 75 |
| Figure 2.2 | Gal4 Assays | 78 |
| Figure 2.3 | hPygo2, β-catenin and BCL9 antisense oligonucleotide and siRNA sequences | 85 |
| Figure 3.1 | Double immunocytochemistry of hPygo2 and β-catenin | 88 |
| Figure 3.2 | Endogenous Wnt activity in Epithelial Ovarian Cancer cell lines | 90 |
| Figure 3.3 | Gal4 Assays | 93 |
| Figure 3.4 | siRNA knockdown studies in TOV-112D EOC cells | 97 |
| Figure 3.5 | Antisense oligonucleotide knockdown studies in TOV-21G EOC cells | 99 |
| Figure 3.6 | Double immunocytochemistry of hPygo2 and CENP-E in synchronized TOV-112D cells | 103 |
## LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AARRS</td>
<td>Al-Awadi/Raas-Rothschild/Schinzel phocomelia syndrome</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer's Disease</td>
</tr>
<tr>
<td>ADPKD</td>
<td>Autosomal Dominant Polycystic Kidney Disease</td>
</tr>
<tr>
<td>APC</td>
<td>Adenomatous Polyposis Coli</td>
</tr>
<tr>
<td>BCL.9</td>
<td>B Cell Lymphoma 9</td>
</tr>
<tr>
<td>BMD</td>
<td>Bone Mineral Density</td>
</tr>
<tr>
<td>BMI</td>
<td>Body Mass Index</td>
</tr>
<tr>
<td>BRCA1</td>
<td>BReast CAnce Type 1 susceptibility protein</td>
</tr>
<tr>
<td>BRCA2</td>
<td>BReast CAnce Type 2 susceptibility protein</td>
</tr>
<tr>
<td>BSO</td>
<td>Bilateral Salpingo-Oophorectomy</td>
</tr>
<tr>
<td>CAD</td>
<td>Coronary Artery Disease</td>
</tr>
<tr>
<td>CamKII</td>
<td>Calmodulin Kinase II</td>
</tr>
<tr>
<td>CAP</td>
<td>cyclophosphamide, doxorubicin and cisplatin</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence Interval</td>
</tr>
<tr>
<td>CGH</td>
<td>Comparative Genomic Hybridization</td>
</tr>
<tr>
<td>CR</td>
<td>Complete Response</td>
</tr>
<tr>
<td>CT</td>
<td>Computed Tomography</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>EOC</td>
<td>Epithelial Ovarian Cancer</td>
</tr>
<tr>
<td>FAP</td>
<td>Familial Adenomatous Polyposis</td>
</tr>
<tr>
<td>FDH</td>
<td>Focal Dermal Hypoplasia</td>
</tr>
<tr>
<td>FEVR</td>
<td>Familial Exudative Vitreoretinopathy</td>
</tr>
<tr>
<td>FZD4</td>
<td>Frizzled 4</td>
</tr>
<tr>
<td>GS</td>
<td>Gardner's syndrome</td>
</tr>
<tr>
<td>HBOC</td>
<td>Hereditary Breast and Ovarian Cancer</td>
</tr>
<tr>
<td>HBM</td>
<td>High Bone Mass</td>
</tr>
<tr>
<td>HCC</td>
<td>Hepatocellular Carcinoma</td>
</tr>
<tr>
<td>HD1</td>
<td>Homology Domain 1</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone Deacetylase</td>
</tr>
<tr>
<td>HNPCC</td>
<td>Hereditary NonPolyposis Colorectal Cancer</td>
</tr>
<tr>
<td>hPygo1</td>
<td>Human Pygopus 1</td>
</tr>
<tr>
<td>hPygo2</td>
<td>Human Pygopus 2</td>
</tr>
<tr>
<td>ICC</td>
<td>ImmunoCytoChemistry</td>
</tr>
<tr>
<td>LRP5</td>
<td>Lipoprotein Receptor-related Protein 5</td>
</tr>
<tr>
<td>LRP6</td>
<td>Lipoprotein Receptor-related Protein 6</td>
</tr>
<tr>
<td>MALDI-TOF</td>
<td>Matrix-Assisted Laser Desorption/Ionization Time Of Flight</td>
</tr>
<tr>
<td>MLH1</td>
<td>Mut L Homologue 1</td>
</tr>
<tr>
<td>MLH2</td>
<td>Mut L Homologue 2</td>
</tr>
<tr>
<td>mM</td>
<td>millimolar</td>
</tr>
<tr>
<td>MM</td>
<td>Mismatch</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix Metalloproteinase</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>NDP</td>
<td>Norrin</td>
</tr>
<tr>
<td>NDS</td>
<td>Normal Donkey Serum</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>NHD</td>
<td>N-terminal Homologous Domain</td>
</tr>
<tr>
<td>OC</td>
<td>Ovarian Cancer</td>
</tr>
<tr>
<td>OC</td>
<td>Oral Contraption</td>
</tr>
<tr>
<td>OODD</td>
<td>Odonto-onycho-dermal Dysplasia</td>
</tr>
<tr>
<td>OPPS</td>
<td>Osteoporosis-pseudoglioma syndrome</td>
</tr>
<tr>
<td>OPS</td>
<td>Osteoporosis-pseudoglioma syndrome</td>
</tr>
<tr>
<td>PCOS</td>
<td>Polycystic Ovary Syndrome</td>
</tr>
<tr>
<td>PCP</td>
<td>Planar Cell Polarity</td>
</tr>
<tr>
<td>PFS</td>
<td>Pregression-Free Survival</td>
</tr>
<tr>
<td>PHD</td>
<td>Plant Homology Domain</td>
</tr>
<tr>
<td>PMS1</td>
<td>Postmeiotic Segregation increased 1</td>
</tr>
<tr>
<td>PMS2</td>
<td>Postmeiotic Segregation increased 2</td>
</tr>
<tr>
<td>PORCN</td>
<td>Human Porcupine gene</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA Interference</td>
</tr>
<tr>
<td>RSP04</td>
<td>R-spondin 4</td>
</tr>
<tr>
<td>SCCOHT</td>
<td>Small cell carcinoma of the ovary, hypercalcemic type</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SERKAL</td>
<td>SEx Reversal, female, with dysgenesis of Kidneys, Adrenals and Lungs</td>
</tr>
<tr>
<td>TAH</td>
<td>Total Abdominal Hysterectomy</td>
</tr>
<tr>
<td>TCF</td>
<td>T Cell Factor</td>
</tr>
<tr>
<td>TCF7L2</td>
<td>Transcription Factor 7-Like 2</td>
</tr>
<tr>
<td>Wg</td>
<td>Wingless</td>
</tr>
</tbody>
</table>
CHAPTER 1
INTRODUCTION

1.1 Ovarian Cancer

1.1.1 Incidence and Epidemiology

Ovarian cancer is the second most common cancer, but the leading cause of death from gynecologic malignancies (Jemal et al, 2008). The American Cancer Society estimates ovarian cancer will account for 21650 or 3% of new cancer cases in American women in 2008, whereas they estimate 15520 or 6% of deaths from cancer in American women (Jemal et al, 2008). That makes ovarian cancer the 7th leading cause of a new diagnosis of cancer in women and the 5th leading cause of death from cancer in American women.

Ovarian cancer incidence rates have slightly decreased over the past 30 years (Jemal et al, 2008). The lifetime risk of ovarian cancer in the general population is 1.39%. This number can be expressed as 1 woman in 72 who will be diagnosed with ovarian cancer sometime in their lifetime (www.seer.cancer.gov accessed September 23, 2008). The age-adjusted incidence rate is 13.3 cases per 100,000 women (www.seer.cancer.gov accessed September 23, 2008). The median age of diagnosis is 63 years old and the median age of death from ovarian cancer is 71 years. The incidence of both diagnosis and death increased with age up to the age of 85 and then declined (www.seer.cancer.gov accessed September 23, 2008). The mean age of diagnosis is younger among women with hereditary or familial disease.
1.1.2 Histopathology

Ovarian tumours display histologic heterogeneity. The World Health Organization (WHO) classifies ovarian tumours based on their histogenesis from the normal ovary. Ovarian cancers can broadly be divided into epithelial and non-epithelial types (see Figure 1.1). The majority of primary ovarian cancer tumours are Epithelial Ovarian Cancers (EOC). They constitute about two thirds of all ovarian neoplasms and an even greater proportion of ovarian malignant neoplasms. They occur predominantly in adults, with the malignant forms generally appearing later in life. They are derived from neoplastic transformation of coelomic epithelial cells on the surface of the ovary and the adjacent ovarian stroma. Nonepithelial ovarian cancers include sex-cord stromal tumours, which are derived from the nongerm cell components of the gonads; germ cell tumours, which are derived from the germ cells; and small cell tumours, which have an unknown origin and are associated with hypercalcemia (Kaku, 2003; Harrison et al, 2006).

Epithelial tumors are classified according to the predominant pattern of differentiation of the tumor cells (Table 1.1). The main histologic types are serous, mucinous, endometrioid, clear cell, transitional cell tumors (Brenner tumors), carcinosarcoma, mixed epithelial tumor, undifferentiated carcinoma, and others (Serov et al, 1973; Kaku et al, 2003). The different histologic types may be associated with different risk factors (Kurian et al, 2005). Stage distribution varies among the types of Epithelial Ovarian Cancer (see Figure 1.2). Serous carcinoma is predominantly found in stage III or IV. On the contrary, clear cell (63%), endometrioid (48%), and mucinous (71%) carcinomas tend to remain confined to the ovary (stage I). The association
Table 1.1

World Health Organization histological classification of ovarian tumors: surface epithelial-stromal tumors (Kaku et al, 2003). Epithelial tumors are classified according to the predominant pattern of differentiation of the tumor cells.
Chapter 1: Introduction

1. Serous tumors
   (1) Benign
      1. Cystadenoma and papillary cystadenoma
      2. Surface papilloma
      3. Adenofibroma and cystadenofibroma
   (2) Of borderline malignancy (of low malignant potential)
      1. Cystic tumor and papillary cystic tumor
      2. Surface papillary tumor
      3. Adenofibroma and cystadenofibroma
   (3) Malignant
      1. Adenocarcinoma, papillary adenocarcinoma, and papillary cystadenocarcinoma
      2. Surface papillary adenocarcinoma
      3. Adenocarcinofibroma and cystadenocarcinofibroma (malignant adenofibroma and cystadenofibroma)

2. Mucinous tumors, endocervical-like and intestinal types
   (1) Benign
      1. Cystadenoma
      2. Adenofibroma and cystadenofibroma
   (2) Of borderline malignancy (of low malignant potential)
      1. Cystic tumor
      2. Adenofibroma and cystadenofibroma
   (3) Malignant
      1. Adenocarcinoma and cystadenocarcinoma
      2. Adenocarcinofibroma and cystadenocarcinofibroma (malignant adenofibroma and cystadenofibroma)

3. Endometrioid tumors
   (1) Benign
      1. Cystadenoma
      2. Cystadenoma with squamous differentiation
      3. Adenofibroma and cystadenofibroma
      4. Adenofibroma and cystadenofibroma with squamous differentiation
   (2) Of borderline malignancy (of low malignant potential)
      1. Cystic tumor
      2. Cystic tumor with squamous differentiation
      3. Adenofibroma and cystadenofibroma
      4. Adenofibroma and cystadenofibroma with squamous differentiation
   (3) Malignant
      1. Adenocarcinoma and cystadenocarcinoma
      2. Adenocarcinoma and cystadenocarcinoma with squamous differentiation
      3. Adenocarcinofibroma and cystadenocarcinofibroma (malignant adenofibroma and cystadenofibroma)
      4. Adenocarcinofibroma and cystadenocarcinofibroma with squamous differentiation (malignant adenofibroma and cystadenofibroma with squamous differentiation)
   (4) Epithelial-stromal and stromal
      1. Adenosarcoma, homologous and heterologous
      2. Mesodermal (müllerian) mixed tumor (carcinosarcoma), homologous and heterologous
      3. Stromal sarcoma

4. Clear cell tumors
   (1) Benign
      1. Cystadenoma
      2. Adenofibroma and cystadenofibroma
   (2) Of borderline malignancy (of low malignant potential)
      1. Cystic tumor
      2. Adenofibroma and cystadenofibroma
   (3) Malignant
      1. Adenocarcinoma
      2. Adenocarcinofibroma and cystadenocarcinofibroma (malignant adenofibroma and cystadenofibroma)

5. Transitional cell tumors
   (1) Brenner tumor
   (2) Brenner tumor of borderline malignancy (proliferating)
   (3) Malignant Brenner tumor
   (4) Transitional cell carcinoma (non-Brenner type)

6. Squamous cell tumors

7. Mixed epithelial tumors (specific types)
   (1) Benign
   (2) Of borderline malignancy (of low malignant potential)
   (3) Malignancy

8. Undifferentiated carcinoma
Figure 1.1

Stage distribution of various types of Epithelial Ovarian Cancers (adapted from Kaku et al, 2003).

A. Staging of ovarian cancer is surgical and based on operative findings. B. Serous carcinoma is predominantly found in stage III or IV, whereas clear cell (63%), endometrioid (48%), and mucinous (71%) carcinomas tend to remain confined to the ovary (stage I). $P < 0.0001$, $\chi^2$-test
A.

Stage I: Growth limited to the ovary
Stage II: Growth involving one or both ovaries with pelvic extension
Stage III: Tumor involving one or both ovaries with peritoneal implants outside the pelvis and/or positive retroperitoneal or inguinal nodes; the tumor is limited to the true pelvis but with histologically verified malignant extension to small bowel or omentum
Stage IV: Growth involving one or both ovaries with distant metastasis; if pleural effusion is present, there must be positive cytological test results to allot a case to stage IV; parenchymal liver metastasis equals stage IV

B.
between endometriosis and EOC varies among the histologic types. Clear cell and endometrioid carcinomas are highly associated with endometriosis (Kaku et al, 2003). Small cell carcinoma of the ovary, hypercalcemic type (SCCOHT) is a rare tumor typically affecting young women. It is an aggressive malignancy with a poor prognosis and few long-term survivors. The histogenesis of SCCOHT and the mechanism of the development of the hypercalcemia are unknown (Harrison et al, 2006).

1.1.3 Pathogenesis

The molecular events leading to the development of epithelial ovarian cancer are unknown. However, there are two main hypotheses that have been proposed. The first is the “incessant ovulation” hypothesis (Fathalla, 1971). It postulates that the ovulation causes repeated minor trauma and repair of the covering epithelium as well as repeated exposure of the ovarian surface to the estrogen rich viscous follicular fluid. This affords an opportunity for genetic mutation and cellular neoplasm. This theory is supported by the total ovulatory years or cycles directly correlating with ovarian cancer risk (Casagrande et al, 1979; Purdie et al, 2003; Moorman et al, 2002; Tung et al, 2005). Risk for ovarian cancer is inversely related to number of pregnancies and lengths of breast-feeding and oral contraceptive use. These events, which interrupt ovulation, are protective factors against developing ovarian cancer.

The second main hypothesis involves persistent exposure of the ovaries to high gonadotropin levels, promoting high estrogen concentrations, leading to epithelial differentiation, proliferation and possibly malignant transformation (Cramer, 1983). The increased gonadotropin levels could be produced by a number of common chemicals and
drugs that may increase gonadotropins by enhancing estrogen degradation in the liver or by directly stimulating production by the pituitary. Alternatively elevated gonadotropins may result from primary ovarian failure, that can result from pelvic irradiation, exposure to chemicals or metabolites toxic to follicles, or ovarian infections such as mumps.

Several other theories have been proposed with varying degrees of supporting evidence. In 1998, Risch proposed a hypothesis for the pathogenesis of ovarian cancer relating to the role of androgens in stimulating epithelial cell proliferation and the protective effects of progesterone. In 2008, a group of Australian scientists studied this hypothesis (Olsen et al, 2008). Using data from an Australia-wide population-based case-control study, they conducted a detailed analysis of ovarian cancer rates in women who used testosterone supplements or the androgenic medication Danazol and in women with factors possibly associated with high circulating levels of androgens, including polycystic ovary syndrome (PCOS), hirsutism and acne. Their results did not support the hypothesis that androgen-related disorders increase the risk of ovarian cancer, with the exception of an increased risk of serous borderline tumours in women who had PCOS. Women who had ever used testosterone supplements had an increased risk of ovarian cancer (OR 3.7; 95% CI 1.1-12.0); however, use of the androgenic medication Danazol did not increase risk (OR 1.0; 95% CI 0.4-2.9).

Another hypothesis is that ovarian inflammation, with rapid DNA turnover, oxidative stress, and increased cytokine production, may play a role in ovarian cancer pathogenesis (Ness RB, Cottreau C, 1999; Ness et al, 2000). Several risk factors for ovarian cancer have some association with inflammatory changes: ovulation entails ovarian epithelial inflammation; talc, endometriosis, cysts, and hyperthyroidism may be
associated with inflammatory responses of the ovarian epithelium. The protective effect of hysterectomies and tubal ligations may be secondary to these gynecologic surgeries precluding irritants from reaching the ovaries via ascension from the lower genital tract. Using TNF-α deficient mice, Balkwill (2000) demonstrated that this proinflammatory cytokine was required for de novo carcinogenesis and that TNF-α is important to the early stages of epithelial tumor promotion. Therefore inflammatory cytokines may not contribute to the genetic damage that initiates the cancer but they may provide the driving force.

1.1.4 Risk and Protective Factors

1.1.4.1 Race and Ethnic Factors

The incidence of EOC varies based on race and geographical location. The incidence rate of ovarian cancer is highest among white and Hawaiian women, intermediate among African-American, Hispanic and Asian-American women, and lowest among Native American women (Daly and Obrams, 1998). From 1986 to 1990, the incident rate among Caucasians was 50% higher than among African American women, both in pre- and post-menopausal populations.

Western countries, including Canada, have high rates of ovarian cancer, approximately three to seven-fold greater than Asian countries and approximately double that of Central/South American countries (Petitti and Potterfield, 1992; Whittemore, 1992; Whittemore et al, 1994). However, the rate of ovarian cancer is higher in Asian immigrants to the Western countries as compared to the rates in Asians overall. This
speaks to the socioeconomic factors that also may play a role in ovarian cancer pathogenesis.

1.1.4.2 Reproductive and Hormonal Factors

In keeping with the “incessant ovulation” and excess gonadotropin theories of pathogenesis, women with a greater number of ovulatory years or cycles are at greater risk of developing ovarian cancer (Casagrande et al, 1979; Purdie et al, 2003; Moorman et al, 2002; Tung et al, 2005). Nulliparity, infertility (RR = 1.36, 95% CI: 1.07, 1.75; Tworoger et al, 2007, see Table 1.2), early age of menarche (before age 12) or late age of menopause (after age 50) increase the number of ovulatory cycles and increase a woman’s risk of ovarian cancer. Whereas risk for ovarian cancer is inversely related to things that inhibit ovulation, such as number of pregnancies, length of breast-feeding and length of oral contraceptive use (Adami et al, 1994; Hankinson et al, 1995; Risch et al, 1983; Rosenblatt et al, 1993; Whittemore, 1992; Whittemore et al, 1994; see Figure 1.3). For women using oral contraceptives for >5 years, the risk ratio for ovarian cancer for ≤20 years since last use was 0.58 (95% confidence interval (CI): 0.39, 0.87), with no association found for >20 years since last use (rate ratio (RR) = 0.92, 95% CI: 0.61, 1.39) (Tworoger et al, 2007).

Low dose OCPs are as or more effective than higher dose OCPs (Lurie et al, 2007). When compared with women who never used hormonal contraception, users of OCPs with low estrogen (equal to or less than 0.035 mg ethinyl estradiol) and low progestin (less than 0.3 mg norgestrel) were at significantly reduced risk of ovarian carcinoma (odds ratio 0.19; 95% confidence interval 0.05-0.75). The risk among these
Figure 1.2

Risk of Invasive Epithelial Ovarian Cancer in US White Women (adapted from Whittemore, 1994; Whittemore et al, 1992). Data sorted according to (A) Number of Term Pregnancies, (B) months of breastfeeding, (C) failed pregnancies (ectopic pregnancies, abortions, miscarriages, and stillbirths), and (D) years of oral contraception (OC) use. Data based on six hospital-based studies and six population-based studies. Odds ratios are adjusted for age (A, B, C, D), parity (B, C, D), and oral contraception use (A, B, C).
Table 1.2

Ovarian Cancer Risk with respect to Contraception use and Infertility (Tworoger et al, 2007). Multivariate relative risk of invasive ovarian cancer among premenopausal Nurses' Health Study participants between 1976 and 2004 (United States), according to history of contraceptive method, other than oral contraceptive pills, and history of infertility.
<table>
<thead>
<tr>
<th>Contraceptive type</th>
<th>No. of cases</th>
<th>No. of person-years</th>
<th>Age-adjusted RR</th>
<th>Multivariate adjusted</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>RR</td>
</tr>
<tr>
<td>No tubal ligation</td>
<td>566</td>
<td>2,041,819</td>
<td>1.00 (ref)</td>
<td>1.00 (ref)</td>
</tr>
<tr>
<td>Tubal ligation</td>
<td>59</td>
<td>457,308</td>
<td>0.63</td>
<td>0.66</td>
</tr>
<tr>
<td>No rhythm method</td>
<td>608</td>
<td>2,395,815</td>
<td>1.00 (ref)</td>
<td>1.00 (ref)</td>
</tr>
<tr>
<td>Rhythm method</td>
<td>17</td>
<td>103,313</td>
<td>0.86</td>
<td>0.77</td>
</tr>
<tr>
<td>No diaphragm</td>
<td>601</td>
<td>2,410,111</td>
<td>1.00 (ref)</td>
<td>1.00 (ref)</td>
</tr>
<tr>
<td>Diaphragm</td>
<td>24</td>
<td>89,016</td>
<td>1.50</td>
<td>1.27</td>
</tr>
<tr>
<td>No condoms</td>
<td>588</td>
<td>2,340,962</td>
<td>1.00 (ref)</td>
<td>1.00 (ref)</td>
</tr>
<tr>
<td>Condoms</td>
<td>37</td>
<td>158,165</td>
<td>1.27</td>
<td>1.10</td>
</tr>
<tr>
<td>No IUD</td>
<td>607</td>
<td>2,434,281</td>
<td>1.00 (ref)</td>
<td>1.00 (ref)</td>
</tr>
<tr>
<td>IUD</td>
<td>18</td>
<td>64,847</td>
<td>1.95</td>
<td>1.76</td>
</tr>
<tr>
<td>No foam</td>
<td>609</td>
<td>2,408,854</td>
<td>1.00 (ref)</td>
<td>1.00 (ref)</td>
</tr>
<tr>
<td>Foam</td>
<td>16</td>
<td>90,273</td>
<td>0.92</td>
<td>0.82</td>
</tr>
<tr>
<td>No vasectomy</td>
<td>579</td>
<td>2,237,864</td>
<td>1.00 (ref)</td>
<td>1.00 (ref)</td>
</tr>
<tr>
<td>Husband's Vasectomy</td>
<td>46</td>
<td>261,264</td>
<td>0.97</td>
<td>0.87</td>
</tr>
</tbody>
</table>

| Infertility                   |             |                     |                 |           |             |
|-------------------------------|-------------|---------------------|-----------------|-----------|
| No                             | 487         | 1,870,756           | 1.00 (ref)      | 1.00 (ref) |
| Yes (Female)                  | 75          | 196,506             | 1.50            | 1.36      | 1.07, 1.75 |
| Yes (Male)                    | 11          | 29,815              | 1.39            | 1.23      | 0.68, 2.25 |
women was lower than among users of estrogen or progestin of high potency, but the difference was not statistically significant.

With OCP being protective, Tworoger et al (2007) studied the effect of other methods of contraception in a large prospective epidemiologic study (US Nurses' Health Study, n=107,900). Intrauterine device use increased the risk of ovarian cancer (RR = 1.76, 95% CI: 1.08, 2.85). Their results suggested other methods of birth control, including the rhythm method, diaphragm, condoms, foam, and vasectomy, did not increase or decrease ovarian cancer risk (see Table 1.2).

Other protective factors include tubal ligations and hysterectomies (Whittemore, 1994; Whittemore et al, 1992). Tubal ligation reduced the risk of ovarian cancer significantly when adjusted for age, oral contraceptive use, parity, and other ovarian cancer risk factors (multivariate relative risk [RR], 0.33; 95% confidence interval [CI], 0.16 to 0.64) (Hankinson et al, 1993). There was a weaker inverse association between simple hysterectomy and ovarian cancer (RR, 0.67; 95% CI, 0.45 to 1.00) (Hankinson et al, 1993). Tubal ligations were found to decrease the rate of ovarian to an even greater extent, by 60% (odds ratio 0.39), in women who carried the BRCA1 Gene (Narod et al, 2001). The protective effect has been postulated to be secondary these procedures limiting the upward migration of irritants and carcinogens through the vagina, cervix, uterus, and fallopian tubes to the ovaries and into peritoneal cavity (Whittemore et al, 1992). Another potential mechanism is that both procedures can impair ovarian blood supply.

With oral contraceptive use being a protective factor prior to the menopause, it might be presumed that postmenopausal hormone replacement therapy may also provide
some protection. However postmenopausal estrogen replacement therapy may increase the risk of ovarian cancer. The American Cancer Society Cancer Prevention Study (Rodriguez et al, 1995) based on mortality data of 224,307 woman-years found an RR of 1.7 (95% CI 1.1–2.8). A 14-year follow-up of this study confirmed these findings with an RR of 1.5 (95% CI 1.2–2.0) for every user and 2.2 (95% CI 1.5–3.2) for use of 10 years or more (Rodriguez et al, 2001). Among former users, the RR decreased with time since last use. The addition of progesterone with estrogen in the hormone replacement therapy regimen has a protective effect and decreases the risk of ovarian cancer to near normal levels. The hazard ratio (HR) for invasive ovarian cancer in women assigned to estrogen plus progestin compared with placebo was 1.58 (95% confidence interval [CI], 0.77-3.24) (Anderson et al, 2003). No appreciable differences were found in the distributions of tumor histology, stage, or grade for either cancer site. The authors concluded that their randomized trial suggested continuous combined estrogen plus progestin therapy may increase the risk of ovarian cancer (Anderson et al, 2003).

A large cohort study suggested that endometriosis is an independent risk factor for epithelial ovarian cancer (van Gorp et al, 2004). They demonstrated a link between endometriosis and endometrioid and clear cell-carcinomas, which could not be explained by shared risk factors alone. The prevalence of endometriosis in mucinous, serous, endometriod, and clear cell ovarian carcinoma was 1.4, 4.5, 19.0, and 35.9%. The risk of malignant transformation of ovarian endometriosis was estimated to be 2.5%. Since endometriosis is a common cause of infertility, this may explain why infertile women are at increased risk of ovarian cancer.
1.1.4.3 Social and Environmental Factors

Cigarette smoking, whether current or in the past, appears to be a risk factor for mucinous ovarian cancer, but not other types of EOC (Jordan et al, 2006; Tworoger et al, 2008). In a meta-analysis of eight population-based case-control studies, one pooled analysis of case-control studies, and one cohort study, Jordan et al (2006) found that the risk of mucinous ovarian cancer doubled in current smokers compared to women who had never smoked (summary RR 2.1, 95% CI 1.7-2.7). There was no increased risk of serous (1.0, 95% CI 0.8-1.2) or endometrioid (0.8, 95% CI 0.6-1.1) cancers and a significant risk reduction for clear cell cancers (0.6, 95% CI 0.3-0.9). The risk of mucinous cancer increased with increasing levels of cigarette smoking. Risk of mucinous ovarian cancer in ex-smokers was 2.02 (CI 1.15-3.55; Tworoger et al, 2008), but returned to that of never smokers within 20-30 years of stopping smoking (Jordan et al, 2006).

A proposed hypothesis for pathogenesis of ovarian cancer is upward migration of a carcinogenic or etiologic agent through the vagina, cervix, uterus and fallopian tubes and into the peritoneal cavity. This is the proposed mechanism of perineal talc increasing the risk of ovarian cancer. There is some data that indicates that talc may be transported retrograde through the fallopian tubes to the ovaries (Wehner, 1994). Some studies have shown an increased risk of ovarian cancer in women exposed to talc (Ness et al, 2000; Harlow et al, 1992), whereas some have not (Gertig et al, 2000). One explanation for the association between talc use and ovarian cancer is that in the past talc has been contaminated with significant amounts of asbestos, a known carcinogen (Cramer et al, 1982).
The role of diet in ovarian cancer pathogenesis is unsettled. Significant heterogeneity and potential biases, even when prospective and controlled, limit interpretation of results. There is no high quality evidence that consumption of any macro- or micro-nutrient, supplement, tea, coffee, or alcohol significantly affects a woman's risk of developing ovarian cancer (Shu et al, 1989; Tworoger et al, 2006; Tworoger et al, 2008; Vainio and Bianchini, 2003; Huncharek and Kupelnick, 2001; Zhang et al, 2002; McCann et al, 2001; McCann et al, 2003; Bosetti et al, 2001; Kushi et al, 1999; Fairfield et al, 2001; Salazar-Martinez et al, 2003; Larsson et al, 2004; Larsson et al, 2006; Bidoli et al, 2001; Giovannucci and Wolk, 2004; Mommers et al, 2006; Koushik et al, 2006; Chang et al, 2007; Prentice et al, 2007; Steevens et al, 2007). There was a slightly increased risk of ovarian cancer in women who had not used oral contraception or post-menopausal hormones (Tworoger et al, 2008). Alcohol intake was not associated with ovarian cancer risk (Tworoger et al, 2008). More data is needed on any potential association between diet and ovarian cancer before any dietary recommendations can be made.

The association between exercise and ovarian cancer is unclear. Multiple case-control studies (Pan et al, 2005; Tavani et al, 2001; Bertone et al, 2002; Bain et al, 1996) and cohort studies (Bertone et al, 2001; Patel et al, 2006; Biesma et al, 2006; Hannan et al, 2004; Weiderpass et al, 2006) have assessed the association of recreational physical activity and ovarian cancer. Two case-control studies (Pan et al, 2005; Bain et al, 1996) showed larger risk reductions in obese women who exercised vigorously. The other studies found no significant effect of physical activity on risk of ovarian cancer. The non-recreational physical activity and epithelial ovarian cancer was assessed in three of
those studies (Pan et al, 2005; Tavani et al, 2001; Patel et al, 2006). Two of the studies (Pan et al, 2005; Patel et al, 2006) found no relationship with risk of ovarian cancer. The other reported a modest inverse association with higher levels of occupational physical activity (Tavani et al, 2001). One study (Anderson et al, 2004) found a positive relationship between vigorous activity and EOC, whereas others have reported no significant association or a small inverse association (Pan et al, 2005; Bertone et al, 2001; Weiderpass et al, 2006; Bertone et al, 2002). Patel et al (2006) reported a significantly increased risk of ovarian cancer with the highest number of hours sitting each day, whereas two other studies (Zhang et al, 2004; Dosemeci et al, 1993) demonstrated no relationship between sedentary activity and EOC.

There appears to be increased risk of ovarian cancer with a high body mass index (BMI). A meta-analysis looked at 28 studies (Olsen et al, 2007). 24 of the 28 studies demonstrated an increased risk of ovarian cancer with obesity, 10 of which reached significance. When averaged in the meta-analysis, the pooled effect estimated for adult obesity was 1.3 (95%, CI1.1-1.5) with a smaller increased risk in overweight individuals (OR1.2; 95%CI1.0-1.3). There was no evidence that the association varied for the different histological subtypes of ovarian cancer. However other papers have the risk as different among different histologic subtypes (Olsen et al, 2008). Obesity was positively associated with clear cell tumors (Odds Ratio 2.3; 95% CI 1.2-4.2) and serous peritoneal tumors (2.9; 1.7-4.9) but not invasive endometrioid, mucinous, or invasive serous tumors overall (0.9; 0.7-1.2). Of the borderline subtypes, obesity was positively associated with serous (1.8; 1.1-2.8) but not mucinous tumors (1.1; 0.7-1.7).
1.1.4 Genetic Factors

Genetic factors are estimated to account for 10 to 15% of ovarian cancer cases (Boyd et al, 2003; Li and Karlan, 2001; Risch et al, 2001; Pal et al, 2005). A personal history of breast cancer, especially at a young age, or a family history of breast or ovarian cancer is one of the strongest risk factors for ovarian cancer, increasing a woman’s risk of EOC two- to six-fold (Schildkraut and Thompson, 1988; Kerlikowske et al, 1992; Bergfeldt et al, 2002).

It is clinically important to distinguish between familial and hereditary EOC. Familial EOC represents a cluster of EOC that occurs based on a combination of environmental and genetic factors. No single gene mutation is responsible. Women with a single family member with ovarian cancer have a 4-5% risk of ovarian cancer, whereas women with two affected relatives have a 7% risk (Daly and Obrams, 1998). Hereditary EOC, defined as having two first-degree relatives with EOC, is caused by a single gene mutation that strongly contributes to the pathogenesis of the EOC. The lifetime risk of developing ovarian cancer in women with a family history of hereditary EOC is 20-50% (Boyd, 2003).

Three hereditary ovarian cancer syndromes exist: the breast and ovarian cancer syndrome; “site-specific” ovarian cancer; and the Hereditary Nonpolyposis Colorectal Cancer (HNPCC) syndrome, also known as Lynch Syndrome. The first two are associated with the BReast CAncer susceptibility proteins (BRCA1 and BRCA2) whereas the HNPCC is associated with a number of germline mutations involving the DNA Mismatch Repair Pathway, primarily MSH2 and MLH1.
1.1.4.4.1 Site-Specific Ovarian Cancer

This syndrome is generally recognized in individuals with two or more first or first and second degree relatives affected with EOC. The lifetime risk is 5%, approximately three fold higher than the general population (Stratton et al, 1998). No specific susceptibility gene has been identified for ovarian cancer. Therefore site-specific ovarian cancer and breast ovarian cancer syndrome are considered to be part of the same spectrum (Pharoah and Ponder, 2002).

1.1.4.4.2 Breast Ovarian Cancer Syndrome

Genetic studies of breast-ovarian cancer syndromes led to the discovery of BReast CAncer susceptibility gene 1 and 2 (BRCA1 and BRCA2). Mutations to either of these genes cause a highly penetrant, autosomal dominant genetic predisposition to ovarian and breast cancer. Founder effects of BRCA1 and BRCA2 mutations have been found in several populations, most notably Ashkenazi Jewish individuals, where 2.5% (1 in 40) carry one of the BRCA mutations (Struewing et al, 1995; Abeliovich et al, 1997). In comparison, 1 in 280 individuals in the general population carry a mutation (Ford et al, 1995).

Mutations to BRCA1 and BRCA2 are responsible for 90% of hereditary ovarian cancer and 10-15% of all ovarian cancer. A meta-analysis of six studies (Takahashi et al, 1995; Matsushima et al, 1995; Stratton et al, 1997; Berchuck et al, 1998; Khoo et al, 2000; Risch et al, 2001) of unselected series of ovarian cancers indicates that 5.7% (70/1236) of all ovarian cancers are associated with germline mutations of BRCA1, while four such studies (Khoo et al, 2000; Risch et al, 2001; Takahashi et al, 1996; Foster et al,
1996) indicate that 3.8% (28/738) of all ovarian cancers are associated with germline mutations in \textit{BRCA2}. The combined figure of 9.5% is likely an underestimate, as all of these studies utilized indirect methods for mutation screening. Subsequent studies, that have used full sequencing of the BRCA genes, have shown BRCA mutations accounting for 15.3% (Pal et al, 2005) and 13.2% (Risch et al, 2006) of ovarian cancer.

The absolute risk of ovarian cancer associated with the presence of BRCA mutations has been investigated by studies worldwide, with the reported estimates varying. A meta-analysis performed in 2007 provides the best estimate of the penetrance of the BRCA mutations (Chen and Parmigiani, 2007). Meta-analytic mean cumulative ovarian cancer risk for mutation carriers at age 70 years was 40\% (95\% CI, 35\% to 46\%) for \textit{BRCA1} and 18\% (95\% CI, 13\% to 23\%) for \textit{BRCA2} mutation carriers. The lifetime risk in the general population was 1.5\%. In a population-based study in Ontario, Canada, the estimated penetrance by age 80 years for carriers of \textit{BRCA1} mutations was 36\% for ovarian cancer and 68\% for breast cancer (Risch et al, 2006).

The risk of ovarian cancer among BRCA heterozygotes is modified by environmental and genetic factors. A rare gene, HRAS1 variable number of tandem repeat (VNTR) locus, has been shown to increase penetrance in \textit{BRCA1} carriers (Phelan et al, 1996). In terms of nongenetic risk modifiers, oral contraceptives and tubal ligation appear to significantly lower the risk of ovarian cancer in \textit{BRCA} mutation carriers (Narod et al, 1998; Narod et al, 2001), consistent with their protective effect in the general population. The protective effects of tubal ligation and oral contraceptive use appear to be additive (Narod et al, 2001).
In women with BRCA mutations who develop cancers, breast cancer is normally diagnosed first (Metcalfe et al, 2005; Liou et al, 2006). The onset of ovarian cancer is related to the mutation type with BRCA1-associated ovarian cancers generally occurring in the fourth decade and BRCA2-associated ovarian cancer occurring in the sixth decade (Karla et al, 2003). In addition to breast and ovarian cancer, BRCA mutation heterozygotes have increased risk of other cancers including prostate cancer in male carriers. However, they do not have an increased risk of developing ovarian tumours of low malignant potential.

Some studies have shown a better prognosis for hereditary ovarian cancer than for the sporadic type (Boyd et al, 2000). In a retrospective cohort study of a consecutive series of 933 ovarian cancers, the hereditary group had a longer disease-free interval following primary chemotherapy in comparison with the nonhereditary group, with a median time to recurrence of 14 months and 7 months, respectively (P<.001) (Boyd et al, 2000). Those with hereditary cancers had improved survival compared with the nonhereditary group (P=.004). For stage III cancers, BRCA mutation status was an independent prognostic variable (P=.03). It has been proposed that this reflects a higher cisplatin sensitivity relative to sporadic cases (Taniguchi et al, 2003; Cass et al, 2003).

Research indicates prophylactic bilateral salpingo-oophorectomy reduces the risk of ovarian and breast cancer (Llort et al, 2007; Finch et al, 2006). The overall (adjusted) reduction in cancer risk associated with bilateral oophorectomy is 80% (multivariate hazard ratio = 0.20; 95% confidence interval, 0.07-0.58; P = .003) (Finch et al, 2006). Bilateral prophylactic mastectomy reduced the risk of breast cancer by approximately
95% in women with prior or concurrent bilateral prophylactic oophorectomy and by approximately 90% in women with intact ovaries (Rebbeck et al, 2004).

1.1.4.4.3 Hereditary Nonpolyposis Colorectal Cancer

Hereditary nonpolyposis colorectal cancer (HNPCC) accounts for 4-6% of colorectal cancer. It can be divided into two categories, Lynch syndromes I and II (Lynch et al, 1991; Watson and Riley, 2005; Watson et al, 2008). Lynch syndrome I is an autosomal-dominant condition characterized by a high risk of early-onset colonic cancer, often proximal and with multiple primaries. Lynch syndrome II includes all the features of Lynch syndrome I along with the addition of other cancers, including endometrial, ovarian, urogenital, and other gastrointestinal primaries. Cumulative risks were highest for colorectal (78%) and endometrial cancers (43%, women only), but ovarian cancer also had an increased lifetime risk (9-12%) (Aarnio et al, 1995). HNPCC cancers account for 1% of ovarian cancers (Rubin et al, 1998).

Lynch syndrome is caused by germline mutations in DNA-mismatch-repair (MMR) genes, predominantly in Mut L Homologue 1 (MLH1), Mut S Homologue 2 (MSH2), and Mut S Homologue 6 (MSH6) and rarely in Postmeiotic segregation increased 1 and 2 (PMS1 and PMS2) (Peltomaki and Vasen, 1997). All these genes are involved in the most important DNA repair mechanisms and are responsible for the repair of the nucleotide mismatch during DNA replication (Arzimanoglou et al, 1996). The MMR genes are located in five different chromosomes and work as heterodimers. The complex, made up of the protein MSH2 with MSH6 or MSH3, recognizes and binds the mismatch during S or G2 phases of cell cycle. MLH1 with PMS1 or PMS2 are involved
in the resynthesis of the DNA strand (Buermeyer et al, 1999). The alteration of this pathway brings about an increased rate of mutations, leading ultimately mutations and dysfunction of growth-regulating genes (Thibodeau et al, 1993).

The recommendations for OC include transvaginal pelvic ultrasound with color Doppler and serum CA125 every 6 months. Hysterosalpingo-oophorectomy should be considered in HNPCC women who undergo surgery for colorectal carcinoma. Prophylactic hysterectomy with bilateral salpingo-oophorectomy is an effective strategy for preventing endometrial and ovarian cancer in the Lynch Syndrome cohort (Schmeler et al, 2006). Schmeler et al (2006) performed a case-control study of 315 women from 1973 to 2004, and found there were no occurrences of endometrial, ovarian, or primary peritoneal cancer among the women who had undergone prophylactic surgery, whereas endometrial cancer was diagnosed in 69 women in the control group (33 percent) and ovarian cancer was diagnosed in 12 women in the control group (5 percent). The main disadvantages of prophylactic hysterectomy and bilateral salpingo-oophorectomy included the surgical complications and premature menopause.

1.1.5 Clinical Manifestations

Most of the Epithelial Ovarian Cancers (EOCs) are diagnosed between 40 to 60 years of age, while non-epithelial ovarian cancers are more common in girls and younger women. Symptoms (the subjective evidence of disease as perceived by the patient) and signs (objective evidence of disease) are similar for all types.

Ovarian cancer is called the “silent killer” because often signs and symptoms are vague and sometimes nonexistent until late in the course of the disease. As well, the
early signs and symptoms are common complaints in the population and have many possible causes, including some benign explanations. This contributes to the difficulty in recognizing and diagnosing ovarian cancer in its early stages. Because of this, clinicians often require a high index of suspicion to make a prompt diagnosis. Ovarian cancer should be considered in the differential diagnosis of any woman presenting with recent onset of abdominal or pelvic complaints, such as bloating, increase abdominal size, urinary urgency or frequency, difficulty eating or feeling full, abdominal pain or pelvic pain. Acute symptoms due to ovarian rupture or ovarian torsion are unusual.

Goff et al (2000) surveyed 1725 women with ovarian cancer. The median age of the women was 52 and 70% had stage III or IV disease. 95% reported symptoms prior to their diagnosis. These symptoms were categorized as abdominal (77%), gastrointestinal (70%), pain (58%), constitutional (50%), urinary (34%), and pelvic (26%). Only 11% of women with Stage I/II and 3% with Stage III/IV reported no symptoms before their diagnosis. The time required for a health care provider to make the diagnosis was reported as less than 3 months by 55%, but greater than 6 months by 26% and greater than 1 year by 11%.

Advanced disease was typically associated with abdominal distension, nausea, anorexia or early satiety due to the presence of ascites and omental or bowel metastases (Eitan et al, 2005). Dyspnea was occasionally present secondary to pleural effusion. Paraneoplastic syndrome and Trousseau’s syndrome are possible, but uncommon.
1.1.6 Physical Exam

With any patient, a thorough physical exam is a necessity. Particular focus must be paid to the abdominal and pelvic exams. Pelvic examination is an extremely sensitive method to detect ovarian cancer; 93% of women with ovarian cancer had palpable masses (Schutter et al, 1998). Therefore it follows that palpitation of an adnexal mass is the usual initial physical sign in a diagnosis of ovarian cancer. However pelvic masses are not specific for ovarian cancer. There are many causes of adnexal masses, including ovarian pathologies such as ovarian cysts, endometriomas, and metastatic carcinomas such as breast, colon and endometrial. Extraovarian masses include ectopic pregnancy, abscesses, cysts, fibroids, inflammatory bowel disease and pelvic kidney.

In premenopausal women only 5-10% of adnexal masses will be malignant. 30-60% of adnexal masses in postmenopausal women are malignant (Roman et al, 1997; Schutter et al, 1998). However, the presence of a solid, fixed pelvic mass is highly suggestive of an ovarian malignancy, with the diagnosis of malignancy almost certain if this fixed, irregular pelvic mass is associated with an upper abdominal mass or ascites.

1.1.7 Laboratory Evaluation

CA-125 is the most well-known ovarian cancer biomarker. It consists of two major domains, A and B, which bind the monoclonal antibodies OC125 and M11, respectively (Nustad et al, 1996). The original clinical assay for CA-125 utilized only the OC125 antibody. The current CA-125 II assay, quantifies CA-125 using both the OC-125 and M11 antibodies (Lloyd et al, 1997).
Table 1.3

<table>
<thead>
<tr>
<th>Screening modality</th>
<th>Stage</th>
<th>Sensitivity, %</th>
<th>Specificity, %</th>
<th>Positive predictive value, %</th>
<th>Negative predictive value, %</th>
<th>Special issues</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA-125</td>
<td>Early stage</td>
<td>50-62</td>
<td>95</td>
<td>57</td>
<td>70.6</td>
<td>Cheap and available in most laboratories. Poor sensitivity in early-stage disease.</td>
</tr>
<tr>
<td></td>
<td>Late stage</td>
<td>90</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ultrasound</td>
<td>All stages</td>
<td>96.8</td>
<td>77</td>
<td>29.4</td>
<td>99.6</td>
<td>Arbitrary cutoff of 3 cm. Low specificity Better suited for differentiation of malignant and benign masses but not for screening.</td>
</tr>
<tr>
<td>Ultrasound with Doppler</td>
<td>All stages</td>
<td>98</td>
<td>87</td>
<td>94</td>
<td>95</td>
<td>Operator dependent. Poor reproducibility. Low specificity.</td>
</tr>
<tr>
<td>Combination U/S and CA-125 (ROC model)</td>
<td>All stages</td>
<td>78</td>
<td>99.9</td>
<td>20.7-26.8</td>
<td>99.9</td>
<td>Better results compared with ultrasound alone or single value of CA-125 with ultrasound.</td>
</tr>
<tr>
<td>LPA</td>
<td>Early stage</td>
<td>90</td>
<td>86.2</td>
<td>93.3</td>
<td>91.8</td>
<td>Nonspecific elevation in ovarian cancer and other gynecologic cancers. No correlation with disease status.</td>
</tr>
<tr>
<td></td>
<td>Late stage</td>
<td>98</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Johns Hopkins group</td>
<td>Early stage</td>
<td>83</td>
<td>94</td>
<td>60</td>
<td>14</td>
<td>Panel of 4 serum tests, not all available at most laboratories. Still below the desired threshold for screening the general population.</td>
</tr>
<tr>
<td>Yale group (6 biomarker panel)</td>
<td>Early stage</td>
<td>91.6</td>
<td>99.4</td>
<td>97.6</td>
<td>98.9</td>
<td>Panel of 6 serum tests, not all available at most laboratories.</td>
</tr>
<tr>
<td></td>
<td>All stages</td>
<td>95.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UCLA group (4 biomarker panel)</td>
<td>Early stage</td>
<td>89</td>
<td>97</td>
<td>97.7</td>
<td>85</td>
<td>Panel of 4 serum tests, all available at most laboratories. Sensitivity and specificity even greater for mucinous ovarian tumors. Still below the desired threshold for screening the general population.</td>
</tr>
<tr>
<td></td>
<td>Late stage</td>
<td>97</td>
<td>99</td>
<td>98.6</td>
<td>98.1</td>
<td></td>
</tr>
</tbody>
</table>
CA-125 is the most studied ovarian cancer biomarker. The research was initiated by the initial findings in the early 1980’s, demonstrating that CA-125 levels greater than 35 U/ml was seen in 83% of patients with epithelial ovarian cancer (East et al, 1983; Canning et al, 1984), whereas the surface epithelium of normal ovaries does not express CA-125 (Kabawat et al, 1983). Further research has revealed that elevated levels of CA-125 were found in greater than 90% of patients with advanced ovarian cancer, but in only 50% of patients with stage I disease (Nustad et al, 1996).

CA-125 has also been shown to have a strong association with serous, rather than mucinous, endometroid and other subtypes of borderline ovarian tumours and ovarian cancers (Hogdoll et al, 2007).

To be an effective screening test for ovarian cancer would require a minimum positive predictive value of 10% and a specificity of 99% (Jacobs et al, 1993; Jacobs et al, 2004; National Institutes of Health consensus Conference, 1995). As a screening tool in the asymptomatic population, CA-125 has inadequate sensitivity (Helzisouer et al, 1993; Skates et al, 2003). In a case-control study in 1993, Helzisouer et al found that the sensitivity of a CA-125 level greater than 35 U/mL within the first 3 years prior to diagnosis of ovarian cancer was 57% (95% confidence interval, 20% to 88%) and the specificity was 100% (95% confidence interval lower limit, 73%). Sensitivity and specificity decreased with increasing time to diagnosis.

There are also a large number of false positive values due to CA-125 serum levels being elevated by another means. A wide variety of conditions elevating serum levels including other cancers (pancreatic, breast, lung, liver, bladder), diverticulosis, liver
cirrhosis, endometriosis, uterine fibroids, benign ovarian lesions, and physiologic conditions such as menstruation and pregnancy. CA-125 is not a useful screening test in premenopausal women because ovarian malignancy is rare at this age and the other causes of CA-125 elevation listed above would be higher in the differential diagnosis. CA-125 is more useful in postmenopausal women, having a 97% positive predictive value (Brooks, 1994; ACOG Committee Opinion Number 280, 2002).

Several studies have looked at the possibility of adding additional novel biomarkers to CA-125 to improve the sensitivity and specificity towards ovarian cancer. A group from John Hopkins studied the combination of CA-125, apolipoprotein A1 (down-regulated in cancer), a truncated form of transthyretin (down-regulated) and a cleavage fragment of inter-α-trypsin inhibitor heavy chain H4 (up-regulated) (Zhang et al, 2004). The Yale group analyzed the combination of biomarkers that included leptin, prolactin, osteopontin, insulin-like growth factor II, macrophage inhibitory factor, and CA-125 (Lester et al, 2007; Visintin et al, 2008). The UCLA group studied four biomarkers (Su et al, 2007). No combination proved to be a sufficient screening test for the general public.

### 1.1.8 Diagnostic Tests

Ultrasound is the most useful non-invasive diagnostic test in women with adnexal masses. It is commonly the first diagnostic imaging performed as it can provide detailed imaging of the ovaries and allows for the detection of sonographic morphologic features of an ovarian mass that may aid in differentiating between benign and malignant ovarian disease. Features suggestive of a malignant diagnosis include: a solid component that is
often nodular or papillary; septations that are greater than 2-3 mm thick; Doppler
demonstration of blood flow into the solid component; presence of ascites; peritoneal
masses; enlarged nodes; and matted bowel.

Computerized tomography (CT) or magnetic resonance imaging (MRI) are useful
in determining local, distant and lymphatic spread of any ovarian cancer and is important
in planning appropriate surgery. Choosing between these two modalities involves taking
into account availability, cost, patient comfort, and radiation exposure.

An ovarian mass may be the result of an extraovarian primary cancer. Therefore
preoperative evaluation would include diagnostic modalities to evaluate the possibility of
cancers that commonly metastasize to the ovary. These include breast, endometrial,
colorectal, gastric, peritoneal and fallopian. Any patient with signs or symptoms of a
gastrointestinal cancer, fecal occult blood or intestinal obstruction should undergo a
barium enema or colonscopy. Both are more sensitive than CT scanning. Upper GI
series is indicated if there are signs or symptoms of an upper GI malignancy.
Mammography should be performed in the presence of any breast mass (Tserkezoglou et
al, 2006).

Image-guided biopsy is a diagnostic option when there is uncertainty as to
whether an ovarian mass has an extraovarian origin or when neoadjuvant chemotherapy
is being considered. Similarly, in patients with ascites, a pathologic diagnosis can often
be made via paracentesis or thoracocentesis.
1.1.9 Management

1.1.9.1 Surgical Management

The initial management of ovarian cancer is almost always surgical. A surgical approach is necessary to obtain tissue to confirm the diagnosis, surgical stage the disease, and attempt optimal cytoreduction. To confirm the diagnosis the abdomen is explored to rule out other primary cancers that have potentially metastasized to the ovary. Surgeons examine the peritoneum, endometrium, fallopian tubes, colon, stomach (for gastric cancer with Krukenberg ovarian metastasis) and breasts.

The stage (see Figure 1.2 for stages) of the disease is assessed surgically. Patients with stage I (confined to the ovary) and Stage II (confined to the pelvis) disease are managed with optimal surgical debulking Young et al, 1983; Young et al, 1990). Systemic chemotherapy may or may not be recommended. Patients with Stage III (disease spread throughout the peritoneal cavity or involving the paraaortic or inguinal lymph nodes) and Stage IV metastatic disease requires aggressive surgical cytoreduction followed by aggressive chemotherapy (Young et al, 1983).

Clinical practice guidelines lay out the procedure for optimal debulking (Morgan et al, 2008). This includes a vertical-incision laparotomy; collection of ascites or peritoneal washings; peritoneal biopsies from the pelvis, paracolic gutters, and suspicious areas from the undersurfaces of the hemidiaphragms; bilateral pelvic and periaortic lymph node dissection if evidence of disease outside the pelvis; omentectomy; total abdominal hysterectomy (TAH); unilateral salpingo-oophorectomy (USO) for stage I disease or bilateral salpingo-oophorectomy (BSO) for higher stage disease; and every attempt to achieve a cytoreduction to less than 1 cm residual disease including a possible
radical pelvic dissection, bowel resection, appendectomy, diaphragm stripping and/or splenectomy.

Many women with ovarian cancer are not receiving recommended comprehensive surgery (Goff et al, 2006; Goff et al, 2007). In a study of 10,432 women with a primary diagnosis of ovarian cancer who underwent at least an oophorectomy, almost half of women with early stage disease were not adequately staged with 21.4% of women receiving no additional staging procedures and 46.8% not have nodal sampling (Goff et al, 2006). In women with advanced disease, the percentage that had additional surgical procedures such as bowel resections was much lower than in institutions that report high optimal cytoreduction rates (Goff et al, 2006). The poor, elderly and minorities were especially susceptible to not receiving recommended comprehensive surgery (Goff et al, 2007). Outcome varying with surgeon specialty (gynecologic oncologists vs obstetrician gynecologists or general surgeons). Patients with stage III ovarian cancer who received surgical treatment by gynecologic oncologists had a 25% reduction in 5-year mortality risk (Junor et al, 1999).

1.1.9.2 Adjuvant Chemotherapy

Therapy for newly diagnosed EOC is dependent on the extent of the disease. The majority of patients, approximately 75%, are diagnosed with stage III (disease that has spread throughout the peritoneal cavity or to neighbouring lymph nodes) or stage IV (metastatic disease). The standard of care for these patients are aggressive surgical debulking followed by systemic chemotherapy (Young et al, 1983).
For the remaining 25% of patients, those with stage I (disease confined to the ovary) and stage II disease (disease outside the ovary, but confined to the pelvis), management begins with surgical cytoreduction (Heintz et al., 2001; Young et al., 1983; Young et al., 1990). No further treatment is recommended for patients with well or moderately differentiated stage I disease (Young et al., 1990). Women with high risk stage I or II EOC benefit from adjuvant chemotherapy. Patients with stage I or II EOC are recommended to have chemotherapy if they have one or more of the following adverse features:

- Stage IA or IB disease with poorly differentiated histology;
- Tumour present on the external surface of the ovary;
- Ruptured tumour capsule;
- Ascites or positive peritoneal washings;
- Stage II disease

The current standard in chemotherapy is paclitaxel plus a platinum-based agent (Bolis et al., 1995; Young, 1975). This has not always been the case, as therapies have and continue to evolve. Platinum compounds, which include cisplatin and carboplatin, are proposed mechanism of action is DNA methylation and disruption of its binding (Taniguchi et al., 2003). In an early study, women with advanced EOC were treated with cyclophosphamide (a mustard alkylating agent), doxorubicin (topoisomerase type II inhibitor and DNA intercalator) plus or minus cisplatin demonstrated the addition of cisplatin increased complete response rate (CR; 51% of patients treated with cisplatin had a normal physical exam, normal radiographic exams and normalization of tumour markers, versus 26% who did not receive cisplatin) and progression-free survival (PFS;
13.1 vs 7.7 months) (Omura et al, 1986). Therefore cyclophosphamide, doxorubicin and cisplatin (CAP) became the standard of care for advanced disease in the 1980’s. A meta-analysis concluded that median survival was improved by 1.91 months with the addition of doxorubicin to the cisplatin/cyclophosphamide regimen, but there was significantly added toxicity (West and Zweig, 1997).

Paclitaxel (Taxol®) was found to be highly active in patients with advanced recurrent EOC clinically resistant to platinum-based chemotherapy (Einzig et al, 1992; Kohn et al, 1994; McGuire et al, 1989; Thigpen et al, 1994). It is proposed to act by promoting microtubule assembly by enhancing the action of tubulin dimers, stabilizing existing microtubules, and inhibiting their disassembly, thus interfering with the late G2 mitotic phase, and inhibiting cell replication. In addition, the drug can distort mitotic spindles, resulting in the breakage of chromosomes. Paclitaxel may also suppress cell proliferation and modulate immune response. The major side effect of paclitaxel was that it produced frequent and severe, albeit manageable, myelosuppression (McGuire et al, 1989; Thigpen et al, 1994). This data led to multiple randomized trials of platinum plus paclitaxel combination chemotherapy versus platinum plus cyclophosphamide. The GOG 111 (McGuire et al, 1996) and OV-10 (Piccart et al, 2000) studies demonstrated significant improvements in overall response, clinical CR, PFS, and overall survival. Eventually in the mid 1990’s, paclitaxel plus a platinum-based chemotherapy became the first line chemotherapeutic treatment.

Phase III trials have failed to demonstrate survival benefit from the addition of a third agent (gemcitabine, liposomal doxorubicin, epirubicin, topotecan, interferon
The choice of second line medical therapy depends on whether the disease was sensitive or resistant to platinum-based chemotherapy. The generally accepted recommendation for women with platinum-sensitive relapse (defined as an objective response to previous platinum-based therapy and a significant relapse-free interval) is a platinum (either cisplatin or carboplatin) plus paclitaxel combination (National Comprehensive Cancer Network guidelines at http://nccn.org/professionals/physician_gls/default.asp). In the management of women with chemoresistant disease single agent therapy is usually chosen. Paclitaxel is the treatment of choice when an objective response has not been achieved with an initial platinum-based regimen (assuming paclitaxel was not part of the regimen). The drugs with the highest response rates in platinum- and paclitaxel-resistant disease are liposomal doxorubicin, oral etoposide, and topotecan (Rose et al, 1998; Muggia et al, 1997; Gordon et al, 2000). Thus, these agents should be considered for initial salvage therapy in women with platinum-refractory disease. Since the reported response rates with all of these drugs is similar, the choice of agent is often driven by the side effect profile and the convenience of administration (Cannistra, 2004).

Because of its relative lack of toxicity, tamoxifen is a reasonable choice in women for whom toxicity is a major concern. Gemcitabine, vinorelbine, leucovorin-modulated 5-FU, and ifosfamide have similar response rates in platinum-resistant patients, but all have a side effect profile that is worse than tamoxifen. Altretamine and oxaliplatin have
minor activity in platinum-resistant disease, but can be considered fourth or fifth-line salvage regimens.

1.1.9.3 Neoadjuvant Chemotherapy

Aggressive cytoreductive surgery followed by adjuvant chemotherapy is the standard of care for advanced-stage ovarian cancer patients. The greatest survival benefit is seen in those with no gross disease left after the initial surgical cytoreduction. However, this represents only 23% of stage III patients and 8% of stage IV patients. Neoadjuvant chemotherapy can decrease tumour load prior to surgery in patients with advanced disease that does not allow for optimal debulking (Schwartz et al, 2008). Neoadjuvant chemotherapy may also be an option for patients with significant comorbidities that prevent or limit surgery or for women who present with massive ascites or poor performance status that may place them at high risk of perioperative mortality.

1.1.10 Prognosis

The prognosis of endothelial ovarian cancer is generally poor. This is primarily due to nature of the symptomatology (symptoms are vague and often absent) and the late presentation of the disease. The majority of patients, approximately 75%, are diagnosed with stage III (disease that has spread throughout the peritoneal cavity or to neighbouring lymph nodes) or stage IV (metastatic disease). Five year survival for these patients who present with stage III or IV disease is less than 50%, dipping to less than one-in-eight patients with stage IV disease surviving 5 years (Heintz et al, 2003). This
Table 1.4

Survival by FIGO stage for patients with ovarian cancer, 1996-98 FIGO statistics

(Heintz et al, 2003).
<table>
<thead>
<tr>
<th>FIGO stage</th>
<th>Number of patients</th>
<th>Overall survival, percent</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 year</td>
</tr>
<tr>
<td>IA</td>
<td>467</td>
<td>98.5</td>
</tr>
<tr>
<td>IB</td>
<td>58</td>
<td>94.7</td>
</tr>
<tr>
<td>IC</td>
<td>560</td>
<td>96.2</td>
</tr>
<tr>
<td>IIA</td>
<td>73</td>
<td>93.1</td>
</tr>
<tr>
<td>IIB</td>
<td>105</td>
<td>91.4</td>
</tr>
<tr>
<td>IIC</td>
<td>206</td>
<td>92.1</td>
</tr>
<tr>
<td>IIIA</td>
<td>120</td>
<td>86.6</td>
</tr>
<tr>
<td>IIIIB</td>
<td>251</td>
<td>86.1</td>
</tr>
<tr>
<td>IIIIC</td>
<td>1653</td>
<td>81.5</td>
</tr>
<tr>
<td>IV</td>
<td>511</td>
<td>64.7</td>
</tr>
</tbody>
</table>
denotes the importance of early diagnosis and the importance of finding diagnostic and therapeutic targets.

1.2 The Wnt Signaling Pathway

The Wnt extracellular signaling pathway (wingless in *Drosophila*) is one of a handful of evolutionarily-conserved signal transduction pathways throughout all of animal development (Cadigan and Nusse, 1997; Wodarz and Nusse, 1998; Hobmayer et al., 2000; Peifer and Polakis, 2000). It functions very similarly in many species, ranging from *Hydra*, a member of the evolutionarily old metazoan phylum Cnidaria (Hobmayer et al., 2000) to *Drosophila*, fruit flies, to the worms *C. elegans* to humans. It stands to the significance of the pathway that it is so conserved in so many species.

Wnt pathways play a key role in normal and malignant development. Wnt signaling is involved in a large number of diverse and varied processes including embryonic development, cell polarity generation, cell fate determination, and cell division/proliferation (reviewed by Cadigan and Nusse, 1997; Moon et al., 1997; Wodarz and Nusse, 1998; Gradl et al., 1999; Moon et al., 2002; van de Wetering et al., 2002).

The Wnt family of signaling ligands consists of a large number of secreted, cysteine-rich, glycoproteins that interact with Frizzled receptors at the cell membrane and activate downstream signaling pathways. There are at least four signaling pathways which includes the planar cell polarity (PCP), JNK, Wnt/Ca\(^{2+}\) and the most widely characterized canonical Wnt pathways (Reviewed by Veeman et al., 2003). Each pathway requires a separate Wnt ligand, has a different downstream signaling cascade, and has different target genes.
1.2.1 Canonical Wnt Signaling

Tight control of the level of β-catenin plays a crucial role in regulating canonical Wnt signaling activity (reviewed by Wodarz and Nusse, 1998). In the absence of Wnt ligand, cytosolic β-catenin is phosphorylated by a complex containing the serine/threonine kinase Glycogen Synthase Kinase 3β (GSK-3β), the tumour suppressor adenomatous polyposis coli (APC), the scaffold protein Axin, along with Diversin and CK1α (Behrens et al., 1998; Ikeda et al., 1998). The phosphorylated β-catenin is targeted by the β-TrCP1 ubiquitin ligase for rapid degradation by the 26S proteosome, thus maintaining β-catenin at minimal levels (Aberle et al., 1997; Hart et al., 1998).

Binding of Wnt to the Frizzled/LRP receptor complex activates the cytoplasmic protein Dishevelled (Dsh; Yanagawa et al., 1995) which, in association with Dapper (Dpr), inhibits the activity of the GSK-3β/APC/Axin/CK1α/Diversin degradation complex (Noordermeer et al., 1994; Kishida et al., 1999; Lee et al., 1999; Smalley et al., 1999). Stabilized β-catenin can then accumulate in the nucleus (Fagotto et al., 1998) where it derepresses Groucho and HDAC-dependent inhibition of Wnt target gene transcription (Cavallo et al., 1998; Roose et al., 1998; Brannon et al., 1999) by forming a complex with T cell factor (TCF), B Cell Lymphoma 9 (BCL9), and pygopus (Behrens et al., 1996; Molenaar et al., 1996; van Noort & Clevers, 2002; Kramps et al., 2002). This transcriptional regulatory complex functions by binding the promoters and recruiting the necessary transcription proteins which activate the expression of downstream target genes such as Myc (He et al., 1998), Cyclin D1 (Shtutman et al., 1999; Tetsu and McCormick, 1999), matrix metalloproteinase MMP-7 (Brabletz et al., 1999; Crawford et al., 1999),
In the canonical Wnt pathway, secreted Wnt ligands initiate intracellular responses by binding to frizzled transmembrane receptors and to low-density lipoprotein (LRP5/6) co-receptors which, in turn, activate the cytoplasmic intermediate, Dishevelled. Dishevelled subsequently binds Axin to inhibit phosphorylation of β-Catenin by the Axin/adenomatous polyposis coli/glycogen synthase kinase-3β tumor suppressor complex (10). Unphosphorylated β-Catenin accumulates in the cytoplasm and nucleus where it interacts with T-cell factor/lymphoid enhancing factor (TCF/LEF), B-cell lymphoma-9 (Bcl-9) and Pygopus (Pygo). Sequence-dependent enhancer binding via TCF/LEF initiates transcription of target genes involved in cell growth and proliferation, such as Cyclin D1 and c-myc.
Chapter 1: Introduction

ITF-2 (Kolligs et al, 2002) and others (for a more complete listing see http://www.stanford.edu/~russe/pathways/targets.html).

1.2.2 Noncanonical Wnt signaling

Originally it was thought that all Wnt signaling occurred through the stabilization and accumulation of β-catenin. Recently two additional Wnt signaling pathways have been discovered (reviewed in Montcouquiol et al. 2006, Strutt 2003, Veeman et al. 2003). In noncanonical Wnt signaling, Wnts still bind to the Fz receptor to activate Dvl, but the downstream signaling cascade does not involve GSK-3β or β-catenin. At least three distinct signaling pathways have been identified: Wnt/Ca\textsuperscript{2+} and Wnt/JNK in vertebrates and Wnt/PCP in Drosophila.

The first indications of the Wnt/Ca\textsuperscript{2+} pathway came with the observation that overexpression of Wnt5a or Wnt11 in Xenopus embryos increased intracellular calcium levels while having no effect on β-catenin levels (Kuhl et al. 2000, Slusarski et al. 1997). The elevated intracellular calcium levels activated protein kinase C (PKC) and calcium/calmodulin-dependent kinase (CamKII) (Kuhl et al. 2000, Sheldahl et al. 1999). The exact mechanism of the pathway’s actions is still unclear. The Wnt/Ca\textsuperscript{2+} pathway is necessary for ventral formation of the Xenopus laevis embryo and in inhibiting the convergent extension of the embryo, which are the morphogenetic movements mediated by the Canonical Wnt pathway (Kuhl 2002).

Polarity of the cell along its apical/basal axis is intrinsic to all cells whether they grow in vitro or in vivo. In essence, all cells have the ability to orientate in the vertical axis. In vivo cells composing most organs must also be organized in a plane
perpendicular to the apical/basal axis. This Planar Cell Polarity (PCP) plays an essential role in the formation of multiple organ systems regulating directed cell migrations, polarized cell division and proper differentiation. The Wnt pathway has been implicated in *Drosophila* (Wnt/PCP) and in vertebrates (Wnt/JNK). Although PCP signaling is not as well understood as the intensively studied canonical pathway it is thought that downstream of Dvl the small GTPases, Rho, Rac and Cdc42 are activated and this leads to regulation of the JNK cascade (Karner et al, 2006; Habas et al, 2003; Habas and He, 2006; Kahn and Moon, 2005).

### 1.2.3 The role of Pygopus in Canonical Wnt signaling

Pygopus is one of the most recently identified proteins in the Canonical Wnt pathway. Humans have two alternately-spliced pygopus mRNAs, both of which are needed for TCF-mediated transcription (Thompson et al., 2002). In *Drosophila*, mutation of *pygopus* causes phenotypes virtually identical to that of wingless (Wg; the homolog of Wnt) and armadillo (Arm; β-catenin’s homolog) knockouts. The Pygopus protein family share two conserved regions, a Plant Homeo Domain (PHD) which binds, through four conserved residues, the Homology Domain 1 (HD1) sequence of BCL9, connecting it to the transcription complex (Townsley et al., 2003; Kramps et al., 2002) and the Pygopus-specific N-homology domain (NHD). PHDs contain a zinc-finger motif which, in other proteins, has been implicated in modulation of chromatin structure, DNA binding and protein-protein interactions (Aasland et al., 1995; Jacobson and Pillus, 1999; Matthews and Sunde, 2002). The N-terminal Homology Domain (NHD) is proposed to recruit cofactors and act as a transcriptional activator (Kramps et al., 2002; Belenkaya et al.,
Figure 1.4

Wnt signaling pathways (Montcouquiol et al, 2006). Vertebrate nomenclature and interactions are listed. (A) The canonical Wnt signaling pathway. In the canonical pathway, binding of Wnt to a Frizzled (Fz) receptor, in association with a coreceptor, either LRP5 or LRP6, leads to activation of Disheveled (Dvl). Dvl inhibits the ability of GSK-3β to target β-catenin (β-cat) for degradation, leading to an increase in cytoplasmic β-cat. Increased cytoplasmic β-cat is translocated to nucleus, where it complexes with members of the TCF/LEF family, leading to changes in transcription. (B) The Wnt-calcium pathway. Binding of Wnt to Fz leads to activation of Dvl, an increase in intracellular calcium and activation of PKC. Increased intracellular calcium can then lead to a secondary activation of PKC as well as to activation of calmodulin kinase II (CamKII). Each of these factors can then mediate various intracellular responses. (C) The Wnt-PCP pathway. Activated Fz receptors lead to activation of Dvl, which then signals through small GTPases and C-Jun N-terminal kinase (JNK) to modulate cytoskeletal elements including actin and microtubules. The PCP pathway also includes a number of other components, such as Vangl, Celsr, and PTK7. The specific roles of these factors have not been fully determined yet, but data from Drosophila suggest that Vangl plays a role in the inhibition or degradation of Dvl. Finally, as indicated by the “?” above Wnt, the specific role of Wnt in activation of the PCP pathway in vertebrates is not fully understood yet.
A Canonical

B Calcium

C PCP

Annu. Rev. Neurosci. 29:363–86
Figure 1.5

The Wnt transcription complex (A) and 3D representation of human pygopus 2 PHD (B). A. β-catenin forms a complex with T cell factor (TCF), B Cell Lymphoma 9 (BCL9), and pygopus (Behrens et al., 1996; Molenaar et al., 1996; van Noort & Clevers, 2002; Kramps et al., 2002). Figure adapted from Kramps et al, 2002. B. 3D representation of hPygo2 PHD.
Chapter 1: Introduction

A

B
Table 1.5

Wnt- and β-catenin-pathway genes that are involved in diseases and syndromes

(adapted from Moon et al, 2004 and


December 21, 2008). Loss of Function (LOF); Gain of Function (GOF).
<table>
<thead>
<tr>
<th>Gene</th>
<th>Condition/Disease</th>
<th>Mutation or Activity/Expression Change</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>WNT1</td>
<td>Schizophrenia</td>
<td>Elevated</td>
<td>Miyaoka et al, 1999</td>
</tr>
<tr>
<td>WNT3</td>
<td>Tetra-amelia</td>
<td>LOF</td>
<td>Niemann et al, 2004</td>
</tr>
<tr>
<td>WNT4</td>
<td>Intersex</td>
<td>GOF</td>
<td>Jordan et al, 2003</td>
</tr>
<tr>
<td>WNT4</td>
<td>Kidney damage</td>
<td>Elevated</td>
<td>Terada et al, 2003</td>
</tr>
<tr>
<td>WNT4</td>
<td>Polycystic Kidney Disease</td>
<td>Variable</td>
<td>Rodova et al, 2002</td>
</tr>
<tr>
<td>WNT4</td>
<td>WNT1A Syndrome</td>
<td>LOF</td>
<td>Mandel et al, 2008</td>
</tr>
<tr>
<td>WNT5a</td>
<td>Metastasis</td>
<td>Elevated</td>
<td>Liang et al, 2003</td>
</tr>
<tr>
<td>WNT5B</td>
<td>Type II diabetes</td>
<td>LOF</td>
<td>Kanazawa et al, 2004</td>
</tr>
<tr>
<td>WNT7A</td>
<td>Fuhrmann syndrome</td>
<td>LOF</td>
<td>Woods et al, 2006</td>
</tr>
<tr>
<td>WNT10A</td>
<td>Odonto-onycho-dermal dysplasia</td>
<td>LOF</td>
<td>Adaimy et al, 2007</td>
</tr>
<tr>
<td>WNT10B</td>
<td>Obesity</td>
<td>LOF</td>
<td>Christodoulides et al, 2006</td>
</tr>
<tr>
<td>sFRP3</td>
<td>Osteoarthritis</td>
<td>SNP; Reduced</td>
<td>Loughlin et al, 2004</td>
</tr>
<tr>
<td>FZ4</td>
<td>FEVR</td>
<td>LOF</td>
<td>Robitaille et al, 2002</td>
</tr>
<tr>
<td>Norrin</td>
<td>FEVR</td>
<td>Low bone mass</td>
<td>Xu et al, 2004</td>
</tr>
<tr>
<td>LRP5</td>
<td>FEVR</td>
<td>LOF</td>
<td>Toomes et al, 2004</td>
</tr>
<tr>
<td>LRP5</td>
<td>High Bone Mass</td>
<td>GOF</td>
<td>Little et al, 2002</td>
</tr>
<tr>
<td>LRP6</td>
<td>early coronary disease</td>
<td>LOF</td>
<td>Mani et al, 2007</td>
</tr>
<tr>
<td>LRP6</td>
<td>late onset Alzheimer</td>
<td>LOF</td>
<td>De Ferrari 2007</td>
</tr>
<tr>
<td>DSH/IVDL</td>
<td>Lung Cancer</td>
<td>Elevated</td>
<td>Uematsu et al, 2003</td>
</tr>
<tr>
<td>APC</td>
<td>Cancer</td>
<td>LOF</td>
<td>van de Wetering, 2002</td>
</tr>
<tr>
<td>AXIN</td>
<td>Cancer</td>
<td>LOF</td>
<td>Giles et al, 2003</td>
</tr>
<tr>
<td>AXIN1</td>
<td>caudal duplication</td>
<td>Undetermined</td>
<td>Oates, 2006</td>
</tr>
<tr>
<td>AXIN2</td>
<td>Tooth Agenesis</td>
<td>LOF</td>
<td>Lammi et al, 2004</td>
</tr>
<tr>
<td>β-catenin</td>
<td>Cancer</td>
<td>GOF</td>
<td>Giles et al, 2003</td>
</tr>
<tr>
<td>β-catenin</td>
<td>Aggressive fibromatosis</td>
<td>Elevated</td>
<td>Cheon et al, 2004</td>
</tr>
<tr>
<td>β-catenin</td>
<td>Pulmonary Fibrosis</td>
<td>Elevated</td>
<td>Chiossi et al, 2003</td>
</tr>
<tr>
<td>TCF7L2 (TCF4)</td>
<td>Type II diabetes</td>
<td>LOF</td>
<td>Grant et al, 2006</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Florez et al, 2006</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>O'Rahilly and Wareham, 2006</td>
</tr>
<tr>
<td>WTX</td>
<td>Wilms tumor</td>
<td>LOF</td>
<td>Major et al, 2007</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Rivera et al, 2007</td>
</tr>
<tr>
<td>PORC1</td>
<td>Focal dermal hypoplasia</td>
<td>LOF</td>
<td>Wang et al, 2007</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Grzeschik et al, 2007</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Leoyklang et al, 2008</td>
</tr>
<tr>
<td>RSPO4</td>
<td>autosomal recessive anonychia</td>
<td>LOF</td>
<td>Bergmann et al, 2006</td>
</tr>
<tr>
<td>VANGLI</td>
<td>Neural tube defects</td>
<td>LOF</td>
<td>Kibar, 2007</td>
</tr>
</tbody>
</table>
Since its discovery, pygopus has been suggested to have a transactivation function (Kramps et al., 2002). For example, when fused to Gal4, pygopus was able to activate transcription from a reporter vector containing a Gal4 binding site (Belenkaya et al., 2002). When overexpressed in Xenopus embryos, the NHD of Pygopus activated expression of the Wnt marker Engrailed-2 and also induced partial secondary body axis development, two results consistent with Wnt activation (Lake and Kao, 2003). When fused to TCF, the NHD was able to activate TOPflash reporter expression (Thompson, 2004). Therefore, pygopus can act as a transcriptional activator with the transactivation function dependent on the NHD.

### 1.2.4 Wnt Signaling and Disease

Along with being involved in normal development, components of the Wnt pathway have been linked to various diseases (see Table 1.5 for a complete list).

#### 1.2.4.1 Gastrointestinal Disease

APC is an enormous protein that has multiple roles in the cell. In the Wnt pathway, it binds to β-catenin in a complex with GSK-3β/ Axin/ CK1α/Diversin and is necessary for β-catenin’s down-regulation. Human APC is a major tumor suppressor gene. Mutations to APC can cause both familial adenomatous polyposis (FAP) and Gardner's syndrome (GS) (Kinzler et al, 1991, Nishisho et al, 1991; Nakamura et al, 1992). FAP and GS are autosomal dominant genetic disorders, which predispose patients to the development of hundreds to thousands of adenomatous polyps in colon and rectum, one
or more of which progress to carcinoma if not surgically treated. Gardner's syndrome is a related disorder in which multiple lipomas, fibromas, osteomas, and desmoid tumors develop as well as a large number of polyps of the colon and rectum. Germ line mutations in APC are responsible for familial FAP and GS (Nakamura et al, 1992). APC is somatically altered by point mutation, deletion or insertion in approximately 85% of sporadic colorectal cancer (Nakamura et al, 1992; Kinzler and Vogelstein, 1996). In all cases, the loss of APC is associated with stabilization of the β-catenin protein in the cytoplasm.

1.2.4.2 Urinary Tract Disease

Several studies have linked alterations in β-catenin regulation to normal kidney development and to kidney disease (Vainio and Uusitalo, 2000; Rodova et al, 2002; Wilson, 2004; Koesters et al, 1999; Zhu, 2000; Kim, 2000) and proper regulation of Wnt signaling is necessary for normal renal development (Vainio and Uusitalo, 2000). For example, there are studies that link β-catenin to proper regulation of the PKD1 promoter (Rodova et al, 2002). PKD1 is mutated in ~85% of patients with autosomal dominant polycystic kidney disease (ADPKD) (Wilson, 2004). Qian et al (2005) created mice carrying a conditional deletion of the Apc tumor suppressor gene specifically in the renal epithelium. As expected, the loss of Apc leads to increased levels of β-catenin protein in renal epithelium. Most of the mice died shortly after birth with multiple kidney cysts. In the rare mouse that survived to adulthood, it had severely cystic kidneys associated with the presence of renal adenomas. Similarly, expression of a form of β-catenin in which the N-terminal 131 amino acids are deleted induces early development of polycystic kidney
disease in a mouse model (Saadi-Kheddouci et al, 2001). These studies confirm an important role for proper regulation of Wnt/β-catenin signaling in renal development and provide evidence that dysregulation of the pathway can initiate tumorigenesis in the kidney.

Mutations in the β-catenin gene have been identified in renal cell carcinomas and Wilms' tumors (Koesters et al, 1999; Zhu, 2000; Kim, 2000; Rivera et al, 2007; Major et al, 2007). Wilms tumor is a pediatric kidney cancer. Rivera et al (2007) used a high-resolution screen for DNA copy-number alterations in Wilms tumors and identified somatic deletions targeting a previously uncharacterized gene on the X chromosome, which they called WTX. WTX is inactivated in approximately one-third of Wilms tumors (15 of 51 tumors). In contrast to biallelic inactivation of autosomal tumor-suppressor genes, WTX is inactivated by a monoallelic "single-hit" event targeting the single X chromosome in tumors from males and the active X chromosome in tumors from females. Major et al (2007) used tandem-affinity protein purification and mass spectrometry to define the protein interaction network of the beta-catenin destruction complex in Wilms' tumours. They found that WTX, a protein encoded by a gene mutated in Wilms tumors, forms a complex with β-catenin, AXIN1, beta-TrCP2 (β-transducin repeat-containing protein 2), and APC (adenomatous polyposis coli). Functional analyses in cultured cells, Xenopus, and zebrafish demonstrate that WTX promotes β-catenin ubiquitination and degradation, which antagonize WNT/β-catenin signaling.
1.2.4.3 Hepatobiliary Disease

Hepatocellular carcinoma (HCC) is the most frequent primary malignancy of the liver. The genetic events responsible for HCC initiation or progression are not clear, but they involve at least three carcinogenesis pathways: the p53, RB, and Wnt/β-catenin signaling pathways (Buendia, 2000). Oncogenic mutations in the β-catenin gene were evident in human and mouse liver tumors (de La Coste, 1998; Miyoshi et al, 1998). β-catenin mutations have been found in 19-41% (average 22%) of human HCCs of different etiologic origin (de La Coste, 1998; Miyoshi et al, 1998; Terris et al, 1999; Tran van Nhieu et al, 1999; Huang et al, 1999; Legoix et al, 1999). The strong correlation between nuclear β-catenin staining and somatic mutations of the β-catenin gene in tumor cells indicates that activation of the Wnt/β-catenin pathway in HCC occurs predominantly through mutations in the β-catenin gene itself (Tran van Nhieu et al, 1999). It differs from colorectal cancers in which APC mutations are responsible for β-catenin stabilization in 70-80% of the cases. HCCs harboring intense nuclear expression of β-catenin are characterized by high proliferative rate, and their prognosis might be more severe (Tran van Nhieu et al, 1999). In benign liver sections, β-catenin immunostaining is restricted to the cell membrane. It has been shown that β-catenin mutations are more prevalent in HCCs that are not related to HBV infection. In APC-knockout mice, 67% of mice developed HCC (Colnot et al, 2004). β-Catenin signaling was strongly activated in these APC-inactivated HCCs.

1.2.4.4 Cardiovascular Disease

Patients with coronary artery disease (CAD) often have accompanying
Table 1.6

Comparison of phenotypes in carriers and noncarriers of \( \text{LRP}_{611C} \) (Mani et al, 2007). Means ± standard deviation are shown for quantitative traits. All kindred members with measured values were included for LDL, triglyceride, HDL, and BMI measurements. For blood pressure, fasting blood glucose, and diabetes, results for subjects over age 40 are shown.
<table>
<thead>
<tr>
<th>Trait</th>
<th>LRP&lt;sub&gt;R611C&lt;/sub&gt; carriers</th>
<th>Noncarriers</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDL (mg/dl)</td>
<td>170 ± 12</td>
<td>98 ± 5</td>
<td>6 x 10&lt;sup&gt;-6&lt;/sup&gt;</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>209 ± 71</td>
<td>68 ± 20</td>
<td>1 x 10&lt;sup&gt;-5&lt;/sup&gt;</td>
</tr>
<tr>
<td>HDL (mg/dl)</td>
<td>57 ± 8</td>
<td>56 ± 7</td>
<td>0.4</td>
</tr>
<tr>
<td>BMI (kg/m&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>24.3 ± 2.6</td>
<td>24.4 ± 1.6</td>
<td>0.13</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>168 ± 21</td>
<td>116 ± 5</td>
<td>8 x 10&lt;sup&gt;-5&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>100 ± 14</td>
<td>81 ± 7</td>
<td>0.0025</td>
</tr>
<tr>
<td>Fasting blood glucose (mg/dl)</td>
<td>159 ± 43</td>
<td>80 ± 3</td>
<td>0.001</td>
</tr>
<tr>
<td>Diabetes (yes/no)</td>
<td>11/4</td>
<td>0/5</td>
<td>0.005</td>
</tr>
</tbody>
</table>
hypertension, diabetes, and aberrant levels of cholesterol and triglycerides. This diverse group of risk factors is collectively known as “metabolic syndrome,” but the underlying molecular mechanisms that link these disorders are still poorly understood. Mani et al. (2007) have identified the causative mutation in a family afflicted with a rare, inherited autosomal dominant form of early-onset CAD with many features of metabolic syndrome and osteoporosis. The culprit was a missense mutation, R611C, in a gene encoding low-density lipoprotein receptor-related protein 6 (LRP6), a co-receptor in the Wnt cellular signaling pathway. R611 lies in an epidermal growth factor (EGF)-like domain (fig. S2) and is conserved among LRP6 orthologs ranging from Xenopus to human.

Analysis revealed complete linkage of LRP6R611C and high LDL, with a lod score of 5.5 (odds of 316,000:1 in favor of linkage). The difference in mean LDL levels between mutation carriers and noncarriers is significant (170 ± 12 mg/dl versus 98 ± 5 mg/dl, P = 6 x 10^-6; Table 1.4). Because high LDL levels are found in all mutation carriers, regardless of age, this trait can serve as a bio-marker of the mutation in subjects too young to manifest CAD. Similarly, LRP6R611C imparts significant effects on triglyceride levels, blood pressure, fasting blood glucose, and prevalence of diabetes (Table 1.4). No significant effects were seen on HDL levels or body mass index. All mutation carriers studied had low bone densities, with values below the 12.5 percentile (P < 0.001).

Mani et al (2007) used NIH3T3 cells to determine the functional significance of the LRP6R611C mutation. In the absence of added Wnt 3a, LRP6R611C showed a 49% reduction of induced signaling compared with that of wild-type LRP6 (P < 0.01). The addition of low doses of Wnt 3a also showed markedly reduced signaling with LRP6R611C.
(42% reduction, \( P < 10^{-5} \)). At high doses of Wnt 3a Wnt signaling through LRP6\(_{R611C}\) does not differ significantly from that of the wild type (\( P = 0.48 \)). Measurement of total LRP6 expression by Western blotting and cell surface expression demonstrate similar levels of wild-type and mutant LRP6. All these findings are consistent with an impaired biochemical function of LRP6\(_{R611C}\) and implicate altered Wnt signaling as a cardiovascular risk factor.

Other studies have demonstrated associations between components of the Wnt pathway with cardiac risk factors. WNT5B (Kanazawa et al, 2004) and transcription factor 7-like 2 (TCF7L2, formerly TCF4; Grant et al, 2006; Florez et al, 2006; O’Rahilly and Wareham, 2006) conferred risk for type 2 diabetes. Kanazawa et al (2004) demonstrated that the \( WNT5B \) gene was strongly associated with type 2 diabetes (\( \chi^2 = 15.6; P = .00008; \) odds ratio = 1.74; 95% confidence interval 1.32–2.29). Expression of the \( WNT5B \) gene was detectable in several tissues, including adipose, pancreas, and liver. In vitro experiments identified the fact that expression of the \( Wnt5b \) gene was increased at an early phase of adipocyte differentiation and overexpression of the \( Wnt5b \) gene in preadipocytes resulted in the promotion of adipogenesis.

Grant et al (2006) demonstrated that a microsatellite, DG10S478, within intron 3 of the transcription factor 7-like 2 gene (TCF7L2; formerly TCF4) was associated with type 2 diabetes (\( P = 2.1 \times 10^{-9} \)). Heterozygous and homozygous carriers of the at-risk alleles had increased risk of Type 2 diabetes as compared to non-carriers (38% and 7% of the population, respectively; RR of 1.45 and 2.41). This corresponds to a population attributable risk of 21%. Common variants in TCF7L2 are associated with an increased risk of diabetes among persons with impaired glucose tolerance with the risk-conferring
genotypes in TCF7L2 being associated with impaired beta-cell function but not with insulin resistance (Florez et al., 2006). The TCF7L2 gene product is a transcription factor previously implicated in blood glucose homeostasis and is thought to act through regulation of proglucagon gene expression in enteroendocrine cells via the Wnt signaling pathway.

WNT10B has been postulated to play a role in the negative regulation of adipocyte differentiation in vitro and in vivo. One proband with early-onset obesity was found to be heterozygous for a missense C256Y mutation (Christodoulides et al, 2006). With the C256Y mutation WNT10B was unable to activate the canonical WNT signaling pathway and block adipogenesis. All relatives of the proband who carried this allele were either overweight or obese. The significance of aberrant Wnt signaling in obesity is still unclear.

1.2.4.5 Neurological and Psychiatric Disease

Alzheimer's disease (AD) is the most common form of age-associated dementia. It is a progressive neurodegenerative disorder characterized by a deficit in cognitive processes manifested as alterations in memory, judgment, and reasoning (Hardy and Selke, 2002). Although the etiology of AD remains to be fully understood, it is well accepted that, along with age, family history is the most prominent risk factor for the development of the disease. Inheritance of the apolipoprotein E-ε4 (APOE-ε4) allele is a risk factor for AD (Corder et al, 1993; Strittmatter et al, 1993), however epidemiological studies estimate that 42–68% of AD patients do not present the APOE-ε4 allele, suggesting that additional genetic or environmental factors could play essential roles in
the disease (Warwick et al, 2000). It has been proposed that altered function of Wnt signaling components may be involved in AD (De Ferrari and Inestrosa, 2000; Mudher and Lovestone, 2002; Caricasole et al, 2003; Moon et al, 2004; De Ferrari and Moon, 2006). Consequently \(LRP6\) gene has been studied in association with this disease. De Ferrari et al (2007) observed that SNP 18e, encoding a C → T missense mutation in \(LRP6\), was strongly associated with AD in the \(APOE-\epsilon4\)-negative patients \((P = 0.0075)\). Individuals carrying at least one copy had a 69–80% greater risk of getting AD compared with individuals being 18e homozygotes.

Most WNTs and FZ receptors are expressed during development of the central nervous system and many are expressed in the adult brain. It has been proposed that increased expression of WNT1 might lead to altered cell adhesion, synaptic rearrangement and plasticity in the brains of people with schizophrenia (Miyaoka et al, 1999). Consistent with the possible involvement of WNT signalling in schizophrenia, Katsu et al (2003) reported FZ3 SNPs are associated with susceptibility to schizophrenia and Kozlovsky et al (2002) have suggested that GSK3\(^\beta\) activity is altered in schizophrenia. Similarly, deletion of Dsh1/Dvl1 in mice produces behavioural defects (Lijam et al, 1999), further linking the WNT pathway to the modulation of brain activity. Inhibition of the Wnt antagonist GSK-3\(^\beta\) by lithium or GSK-3\(^\beta\) antisense short hairpin RNA (shRNA) provides protects neurons from death (Hongisto et al, 2008).

Neural-tube defects such as anencephaly and spina bifida (myelomeningocele) are common congenital malformations affecting 1-2 babies per 1000 (Copp et al, 2003). Neural tube defects occur because of partial or complete failure of the neural tube to close during embryogenesis. Pathogenesis includes complex genetic and environmental

\textbf{1.2.4.6 Musculoskeletal Disease}

Osteoporosis-pseudoglioma syndrome (OPPG; alternatively known as OPS and osteogenesis imperfecta, ocular form) is an autosomal recessive disorder generally characterized by congenital or infancy-onset visual loss and skeletal fragility recognized during childhood. Mutations in the low-density lipoprotein receptor-related protein 5 (encoded by \textit{LRP5}) cause OPPG (Gong \textit{et al}, 2001). OPPG is a rare disorder, with an estimated population incidence of 1 per 2,000,000 and a carrier frequency of 1 per 700. Heterozygous carriers of OPPG-causing mutations have reduced bone-mineral density
(BMD) compared with age- and sex-matched controls (Gong et al, 2001; Lev et al, 2003) and \textit{LRP5} mutations have been found among individuals with "idiopathic" osteoporosis and/or skeletal fragility (Hartikka et al, 2005). Two other phenotypes have been attributed to a mutation in \textit{LRP5}. Heterozygous missense mutations in the receptor’s first six-bladed propeller domain can cause autosomal dominant disorders of high bone mass (HBM), in which BMD is several SDs above the mean (Boyden et al, 2002; Little et al, 2002; Van Wesenbeeck et al, 2003). These mutations may cause a gain of function in the receptor by altering a binding site for the receptor’s endogenous inhibitors (Boyden et al, 2002; Ai et al, 2005). Heterozygous and homozygous mutations have also been described in some patients with the eye disease familial exudative vitreoretinopathy (FEVR; Jiao et al, 2004; Toomes et al, 2004; Qin et al, 2005). FEVR can also be caused by mutations in a secreted ligand, Norrin (NDP; Chen et al, 1993; Xu et al, 2004), and an \textit{LRP5} coreceptor, Frizzled 4 (FZD4; Robitaille et al, 2002; Qin et al, 2005; Xu et al, 2004).

WNT signaling plays a role in vertebrate limb development (Yang, 2003). In the developing vertebrate limb, Wnt signaling is required for limb bud initiation, early limb patterning (which is governed by several well-characterized signaling centers), and late limb morphogenesis events. Split-hand/split-foot malformation (SHFM) is a limb malformation involving the central rays of the autopod and presenting with syndactyly, median clefts of the hands and feet, and aplasia and/or hypoplasia of the phalanges, metacarpals, and metatarsals. Some patients with SHFM1 have been found to have mental retardation, ectodermal and craniofacial findings, and orofacial clefting (Elliott and Evans, 2006). A definite autosomal recessive inheritance for isolated SHFM has
been reported for one family only (Gul and Oktenli, 2006; Ugur and Tolun, 2008). Ugur and Tolun (2008) studied a large consanguineous family afflicted with autosomal recessive SHFM. Twelve affected members had central feet reductions with or without hand involvement. They identified a homozygous C to T missense WNT10b mutation (p.R332W) in all affected individuals but the atypical case plus in an asymptomatic female.

Anonychia is an autosomal recessive disorder characterized by the congenital absence of fingernails and/or toenails. It is a rare entity that may present either as a so-called partial autosomal dominant form that affects only the thumbs (Strandoskow, 1939) or, in its most severe, autosomal recessively inherited variant, with involvement of all digits and toes (Littman and Levin, 1964). Bergmann et al (2006) studied a large German nonconsanguineous family with four affected and five unaffected siblings with isolated total congenital anonychia. They performed genomewide mapping and showed linkage to 20p13. Analysis of the RSPO4 gene within this interval revealed a frameshift and a nonconservative missense mutation in the highly conserved exon 2. Both mutations were not present among controls. Blaydon et al (2006) studied eight affected families and identified homozygous or compound heterozygous mutations in the gene encoding R-spondin 4 (RSPO4). RSPO4 expression was specifically localized to developing mouse nail mesenchyme at embryonic day 15.5, suggesting a crucial role in nail morphogenesis. RSPO4 is a member of the recently described R-spondin family of secreted proteins that play a major role in activating the Wnt/ beta -catenin signaling pathway (Kim et al, 2006; Nam et al, 2006).
Tetra-amelia is a rare human genetic disorder characterized by complete absence of all four limbs and other anomalies. Niemann et al (2004) studied a consanguineous family with four affected fetuses displaying autosomal recessive tetra-amelia and craniofacial and urogenital defects. They identified a homozygous nonsense mutation (Q83X) in the WNT3 gene in affected fetuses of the family. The Q83X mutation truncates WNT3 at its amino terminus, suggesting that loss of function is the most likely cause of the disorder. The identification of a WNT3 mutation in tetra-amelia indicates that WNT3 is required at the earliest stages of human limb formation and for craniofacial and urogenital development.

In a Turkish-Arabian family, Fuhrmann et al (1980) initially described a 'new' syndrome consisting of bowing of the femurs, aplasia or hypoplasia of the fibula, and poly-, syn-, and oligodactyly. Other findings included hypoplasia of pelvis, congenital dislocation of hips, absence or coalescence of tarsal bones, absence of various metatarsals, hypoplasia of fingers and fingernails, and postaxial polydactyly. In a Jordanian family, Al-Awadi et al (1985) described a more severe syndrome with deficiency of all 4 extremities. Two family members had hypoplastic femora and absent ulnae and fibulae. Although 'thoracic dystrophy,' pelvic deformity, and unusual facies were mentioned, these did not seem as impressive as the limb malformations. In the Pakistani Muslim family described by Kumar et al (1997), Woods et al (2006) found that affected individuals had 3 homozygous changes, all in exon 3 of the WNT7A gene: a missense mutation, 630G-A, that led to an alanine-to-threonine substitution (A109T), and 2 known synonymous SNPs. The results suggest that a partial loss of WNT7A function causes Fuhrmann syndrome (and a phenotype similar to mouse Wnt7a knockout),
whereas the more-severe limb truncation phenotypes observed in Al-Awadi/Raas-Rothschild/Schinzel phocomelia syndrome (AARRS) result from null mutations (and cause a phenotype similar to mouse Shh knockout).

Lammi et al (2004) studied a Finnish family in which severe permanent tooth agenesis (oligodontia) and colorectal neoplasia segregate with dominant inheritance. Eleven members of the family lacked at least eight permanent teeth, two of whom developed only three permanent teeth. Colorectal cancer or precancerous lesions of variable types were found in eight of the patients with oligodontia. They found that affected family members had a nonsense mutation, Arg656Stop, in the Wnt-signaling regulator AXIN2. They also identified a de novo frameshift mutation 1994-1995insG in AXIN2 in an unrelated young patient with severe tooth agenesis. As Axin is an inhibitor of Wnt signaling, both mutations are expected to activate the signaling pathway. The results suggest Wnt signaling is important for the development of dentition.

1.2.4.7 Genital and Reproductive Anomalies

At 6 weeks of human development, the male and female genital systems are indistinguishable in appearance. It consists of two sets of paired ducts: the paramesonephric (müllerian) ducts and the mesonephric (wolffian) ducts. In the absence of the testis-determining factor of the Y chromosome, the mesonephric ducts begin to degenerate. At the same time the paramesonephric ducts develop proximally to form the fallopian tubes and distally give rise to the uterus and upper four-fifths of the vagina. WNT4 suppresses male sexual differentiation by means of the regulation of müllerian-duct formation and control of ovarian steroidogenesis (Vainio et al, 1999; Jordan et al,
2001; Jeays-Ward et al, 2003). In a mouse model in which Wnt4 is ablated, both male and female Wnt4-knockout mice have defects in kidney development and adrenal function. The female mice exclusively had deficiencies in gonadal development and steroidogenic function (Vainio et al, 1999; Stark et al, 1994). Biason-Lauber et al (2004) presented a case report of an 18-year-old woman presented with primary amenorrhea. Further examination revealed müllerian agenesis, unilateral renal agenesis, and clinical signs of androgen excess—a phenotype resembling the Mayer-Rokitansky-Küster-Hauser syndrome and remarkably similar to that of female Wnt4-knockout mice. A genetic evaluation revealed a loss-of-function mutation in the WNT4 gene.

Mandel et al (2008) studied a consanguineous kindred of Arab Muslim origin. The family had been followed since the birth of an infant who died of type 1 citrullinemia at age 4 days. The parents were second cousins. Three affected fetuses showed largely overlapping features including female sex reversal and dysgenesis of kidneys, adrenals, and lungs, given the acronym of SERKAL syndrome (SEx Reversal, female, with dysgenesis of Kidneys, Adrenals and Lungs). In the case of all 3 affected fetuses, pregnancy was terminated because of the finding of renal agenesis. Given the similarities between the findings in these cases and a WNT4 knockout mouse model (Vainio et al, 1999; Heikkila et al, 2005), Mandel et al (2008) genotyped all available family members for microsatellite markers spanning the WNT4 locus. The affected fetus from which DNA was available displayed a homozygous haplotype, which was found to be carried in heterozygous state by all parents and unaffected sibs, suggesting the existence of a homozygous mutation in WNT4 in the affected fetus. The mutation was found to be a homozygous transition resulting in the amino acid substitution A114V. The mutation
resulted in markedly reduced WNT4 mRNA levels in vivo and in vitro and downregulated WNT4-dependent inhibition of beta-catenin degradation.

Caudal duplication anomaly was initially coined by Dominguez et al (1993) to describe the occurrence of duplications of different organs in the caudal region. They reported 6 affected patients and reviewed 8 similar previously published cases. Caudal duplication anomaly is similar to an anomaly seen in Axin(Fu) mice, which carry a mutation in the Axin locus (Vasicek et al, 1997). Affected mice display bifurcated tails as a result of caudal duplication in the distal region. Axin encodes an inhibitor of the Wnt-signaling pathway and has been shown to regulate embryonic axis formation in mouse and in Xenopus (Zeng et al, 1997). Suppression of wildtype Axin in Xenopus embryos results in the duplication of the body axis. Using bisulfite sequencing, Oates et al (2006) examined methylation at the promoter region of the AXIN1 gene in the MZ twins discordant for a caudal duplication anomaly in whom no causative AXIN1 mutation was found (Kroes et al, 2002). Methylation of the promoter region in peripheral blood mononuclear cells was variable among individuals, including MZ pairs. In the MZ pair discordant for the caudal duplication, this region of the affected twin was significantly more methylated than that of the unaffected twin (P less than 0.0001), which was significantly more methylated than those of the controls (P = 0.02). Oates et al (2006) confirmed that this CpG island functions as a promoter in vitro and that its activity is inversely proportional to the extent of methylation. This finding raised the possibility that hypermethylation of the AXIN1 promoter, by mechanisms as yet undetermined, is associated with the malformation.
1.2.4.8 Dermatologic Disease

Focal Dermal Hypoplasia (FDH) is inherited as an X-linked dominant multisystem birth disorder affecting tissues of ectodermal and mesodermal origin. Affected males die in utero. Affected female patients have features including atrophy and linear pigmentation of the skin, herniation of fat through the dermal defects, and multiple papillomas of the mucous membranes or skin. In addition, patients can also present with digital anomalies consisting of syndactyly, polydactyly, camptodactyly, and absence deformities; oral anomalies; lip papillomas; hypoplastic teeth; ocular anomalies (coloboma of iris and choroid, strabismus, microphthalmia); mental retardation; and striated bones (Larregue and Duterque, 1975; Happle and Lenz, 1977).

Wang et al (2007) performed a genomewide oligonucleotide comparative genomic hybridization (CGH) array and identified a 219-kb deleted region in 2 affected individuals. This region encompassed 5 genes including PORCN. Grzeschik et al (2007) used CGH to study 6 affected members of 1 family segregating FDH and 10 unrelated affected individuals. They identified a similar deletion in Xp11.23 that overlapped by approximately 80 kb and included 4 genes, including PORCN. Wang et al (2007) amplified and sequenced all coding exons of the PORCN gene and identified heterozygous mutations in 10 FDH-affected women. In 8 individuals with FDH, Grzeschik et al (2007) identified several heterozygous nonsense mutations and 1 splice site mutation. Leoyklang et al (2008) reported 3 unrelated Thai girls with sporadic FDH in whom they identified mutations in the PORCN gene, confirming that PORCN is the gene responsible for FDH across different populations.
Odonto-onycho-dermal dysplasia (OODD) is a rare autosomal recessive syndrome in which the presenting phenotype is dry hair, severe hypodontia, smooth tongue with marked reduction of fungiform and filiform papillae, onychodysplasia, keratoderma and hyperhidrosis of palms and soles, and hyperkeratosis of the skin. OODD was originally reported in 3 consanguineous Lebanese Muslim Shiite sibships by Fadhil et al (1983). Arnold et al. (1995), Zirbel et al. (1995), and Megarbane et al. (2004) have described 11 additional affected patients. Adaimy et al (2007) studied 3 consanguineous Lebanese Muslim Shiite families that included 6 individuals affected with odontoonychodermal dysplasia. They found homozygosity for the same nonsense mutation in the WNT10A gene (E233X). The mutation was predicted to result in a prematurely truncated protein of 232 amino acids instead of 417 amino acids.

1.3 Thesis Rationale

Ovarian cancer is called “the silent killer”. Current therapies have had limited success with increasing survival and curing these patients. Previous research in our laboratory demonstrated overexpression of Pygopus in several EOC cell lines (Popadiuk et al, 2006). Immunoblots of TOV-112D and TOV-21G demonstrated a large increase in pygopus expression compared to the Immortalized Ovarian Surface Epithelial cell line (IOSE 397; a kind gift from Dr. Nelly Auersberg, University of British Columbia, Canada; McNeish et al, 2005; Xu et al, 2006). In contrast, β-catenin was only overexpressed in TOV-112D cells and was expressed minimally in TOV-21G cells. The co-overexpression of pygopus in TOV-112D cells is consistent with a requirement for these proteins in deregulated canonical Wnt signaling. TOV-21G cells only
overexpressed pygopus. This indicates that pygopus may have a more central role in cancer development and proliferation, and that this role is independent of β-catenin. In my research, I have explored the possibility of pygopus as a unique and novel therapeutic target for ovarian cancer. With pygopus overexpression limited to a select number of tissues, including the ovaries, pygopus suppression could target the ovarian cancer cells selectively with theoretically fewer side effects and effect on other organ systems.

While current evidence suggests that Pygopus is dedicated to the Wnt pathway, it is possible that it also has Wnt-independent functions, as described (Song et al, 2007; de la Roche and Bienz, 2007). To demonstrate this possibility in cancer, I studied the role of Pygopus in two different epithelial ovarian cancer cell lines (TOV-112D and TOV-21G), only one of which (TOV-112D) expressed β-catenin. My results indicated that while canonical Wnt signaling could be demonstrated in only in the β-catenin-expressing cell line, both cell lines overexpressed and required pygopus for growth, clearly suggesting that, as in embryonic development, pygopus has activity independent of wnt signaling in EOC.
Chapter 2: Materials and Methods

Chapter 2
MATERIALS AND METHODS

2.1 Cell Culture

The previously characterized epithelial ovarian cancer (EOC) cell lines, TOV-112D and TOV-21G (Provencher et al., 2000), SK-OV-3 (Fogh, 1975; Fogh et al., 1977) and the NIH:OVCAR-3 cell line (Hamilton et al, 1983) were obtained from the American Tissue Culture Collection (ATCC; CRL-11730, CRL-11731, HTB-77 and HTB-161, respectively). All three cell lines were cultured in Delbecco’s Modified Eagle’s Medium (DMEM; Gibco BRL) containing 10% fetal calf serum (FCS), 100 U/mL of penicillin and 100μg/mL of streptomycin. Cells were passaged every three to four days.

TOV-112D cells have a known activating mutation in β-catenin (Wu et al, 2001) that results in overexpression of β-catenin and increased canonical Wnt activity. In comparison, TOV-21G cells have minimal expression of β-catenin (Popadiuk et al, 2006). SK-OV-3 and OVCAR-3 cell lines have moderate β-catenin expression (Popadiuk et al, 2006). TOV-21G, SK-OV-3 and OVCAR-3 have baseline canonical Wnt activity (Popadiuk et al, 2006).

The SV40 Immortalized Ovarian Surface Epithelial 397 (IOSE 397) cell line was a gift from Dr. Nelly Auersperg on behalf of the Canadian Ovarian Tissue Bank. Cells were cultured in a 1:1 combination of two media, 199 (Sigma) and MCDB105 (Sigma) with 5% FCS, 100 U/mL of penicillin and 100μg/mL of streptomycin. Cells were passaged every three to four days.
2.2 TCF-dependent Transcription Reporter Assays

To measure canonical Wnt activity in the EOC cell lines, TCF-dependent transcription reporter assays were performed as previously described (Morin et al., 1997). 2 x 10^5 cultured cells were seeded in 6-well plates 24 hours prior to transfection. Cells were transfected with 0.4 μg of either pTOPflash or pFOPflash (Upstate Biotechnology) using Effectene® Transfection Reagent (Qiagen). Forty-eight hours post-transfection, cells were washed with PBS and lysed with the supplied Luciferase Cell Culture Lysis Buffer (Promega). Luciferase Assay Reagent was added and the light produced was measured with a Monolight® 2010 Luminometer (Analytical Luminescence Laboratory, San Diego, CA). Each experiment was performed in triplicate.

To normalize transfection efficiency 0.4 μg of the RSV β-gal vector was co-transfected into the cells. 200 μL of Z buffer (16.1 g/L Na_2HPO_4 · 7H_2O, 5.5 g/L NaH_2PO_4 · H_2O, 0.75 g/L KCl, 0.25 g/L MgSO_4 · 7H_2O, and 0.27% β-mercaptoethanol) was added to 20 μL of cell lysate and incubated at 37°C for 15 minutes. 100 μL of 1 M Na_2CO_3 was added to stop the reaction. β-galactosidase levels were relative to the optical density obtained at 420 nm using an Ultrospec 2000 UV/Visible Spectrophotometer (Pharmacia Biotech).

2.3 Plasmids

Mammalian hPygo2 expression constructs were produced by subcloning hPygo2 from pOTB7/hPygo2 (engineered by Dr. B. Lake) and insertion into pCS2+ (a gift from Dave Turner). The full length hPygo2 construct (pCS2+/hPygo2) was prepared by releasing the hPygo2 insert from pOTB7/hPygo2 and ligating it into the EcoRI and XhoI
Chapter 2: Materials and Methods

TOPflash Mechanism. To determine the amount of endogenous Wnt signaling in the ovarian cancer cell lines a TOPflash assay was performed. The ovarian cancer cells had either the TOPflash or the control FOPflash vector transfected into them. The TOPflash vector had six TCF binding sites allowing endogenous TCF to bind and recruit the remainder of the Wnt transcription complex. This complex activates the expression of luciferase. Luciferase Assay Reagent was added and the light produced was measured with a Monolight® 2010 Luminometer (Analytical Luminescence Laboratory, San Diego, CA). The FOPflash vector was used as a control. It had six mutated TCF binding sites. TCF is not able to bind. Therefore it is not able to recruit the transcription complex and luciferase is not expressed. Each experiment was performed in triplicate.
Chapter 2: Materials and Methods

FOPflash mutant TCF binding site

TOPflash wildtype TCF binding site

TK prom

Luciferase

TK prom

Luciferase
restriction sites of pCS2+. The full length antisense construct (pCS2+/as-hPygo2) was prepared by the digestion of pCS2+/hPygo2 with BamHI and CiaI restriction enzymes and ligating the released fragment into the BamHI and CiaI sites of pCS2+. All plasmids were sequenced with the USB Sequencing Kit (Amersham) to confirm specificity.

Gal-4-hPygo2 fusion proteins were constructed by PCR amplification of different regions of hPygo2 (see Figure 3.3A) followed by the insertion into the EcoRI and XhoI restriction sites of pCMV-Tag2b (Stratagene). hPygo2 inserts were then subcloned from pCMV-Tag2b into the EcoRI and HindIII restriction sites of pMG4 to make N-terminal Gal-4-hPygo2 fusion proteins. All plasmids were sequenced with the USB Sequencing Kit (Amersham) to confirm correct insert sequences.

2.4 Gal4-fusion Transcription Assays

2 x 10⁵ cultured cells were seeded in 6-well plates 24 hours prior to transfection. Using the Effectene® Transfection Reagent (Qiagen) 0.15 μg of each of the pFR luc reporter, a pM pygopus vector, and the RSV β-gal control plasmid were transfected into TOV-21G and TOV-112D cells. After 48 hours cells were washed with PBS and protein was extracted in Luciferase Cell Culture Lysis Buffer (Promega). Luciferase and β-galactosidase reporter expression was measured as previously described. Luciferase values were also normalized by total protein as determined by BioRad® assays.

2.5 Protein Extraction and Western Immunoblots

Protein was extracted directly in SDS PAGE sample buffer from cells on 80% confluent plates and homogenized several times through a 21G syringe. 20 μL of protein
Figure 2.2

**Gal4 Assays.** To determine the amount of transactivation activity of hPygo2 in the presence and absence of the Wnt pathway, Gal4 Assays were performed. The hPygo2-Gal4 Binding Domain fusion protein binds the Gal4 binding sites, and the transactivation activity of hPygo2 is measured by luciferase expression.
Chapter 2: Materials and Methods

Nucleus

Gene X
Gal4
BD

Transcription Machinery

Gal4 Binding Sites

Reporter Gene
Table 2.1

Primer Sequences and PCR Conditions for Gal-4-hPygo2 Constructs.

<table>
<thead>
<tr>
<th>Primer</th>
<th>PCR Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>TA</td>
<td>Primer annealing temperature.</td>
</tr>
<tr>
<td>N</td>
<td>Number of annealing/elongation cycles.</td>
</tr>
</tbody>
</table>

Synthetic nuclear localization sequence is in **bold**. Synthetic stop codon is *underlined*
<table>
<thead>
<tr>
<th>Construct</th>
<th>Upstream primer (5'-3')</th>
<th>Downstream primer (5'-3')</th>
<th>TA</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gal-4-hPygo2-1</td>
<td>GTCCCCCACTCCATG-</td>
<td>TCAGCCAGGGGGTG-</td>
<td>60</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>GGCCGCCTCG</td>
<td>CCAAGCTGTTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gal-4-hPygo2-2</td>
<td>ATGGGCTCCAAAGAAGA-</td>
<td>TCACCCCATCGTTAGC-</td>
<td>60</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>AAGCGTAAGGTACAG-</td>
<td>AGCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CCTCCCCCAGGCTTGG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gal-4-hPygo2-3</td>
<td>ATGGGCTCCAAAGAAGA-</td>
<td>TCAGCCAGGGGGTG-</td>
<td>60</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>AGCGTAAGGTACAG-</td>
<td>CCAAGCTGTTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AACCCTTTTGAAGATGAC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gal-4-hPygo2-4</td>
<td>GTCCCCCACTCCATG-</td>
<td>CCAAGGAATGGAGG-</td>
<td>65</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>GCCGCCTCG</td>
<td>GGCTGCAAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gal-4-hPygo2-5</td>
<td>ATGAAGAGTCCAGAA-</td>
<td>CCAAGGAATGGAGG-</td>
<td>65</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>AAGAAGAC</td>
<td>GGCTGCAAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gal-4-hPygo2-6</td>
<td>ATGAAGAGTCCAGAA-</td>
<td>TCACCCCATCGTTAGC-</td>
<td>60</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>AGAAGC</td>
<td>AGCC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
lysate was run on 10% SDS-PAGE protein gels and transferred to Hybond™ ECL™ nitrocellulose membranes under semi-dry conditions. Western immunoblots were performed essentially as previously described (Popadiuk et al., 2006). Proteins were immunodetected using the ECL™ Western Blotting Detection System and Hyperfilm™ (Amersham Pharmacia Biotech). Rabbit polyclonal anti-pygopus antisera was developed previously (Popadiuk et al., 2006). Mouse monoclonal anti-β-catenin antibodies and rabbit polyclonal anti-ERK antisera were purchased from Santa Cruz. Mouse monoclonal anti-β-actin antibodies were purchased from Sigma.

2.6 Co-immunoprecipitations

Cells were grown on 6-well plates until 80% confluent. Cells were washed with cold PBS. Protein was extracted in lysis buffer (1 mg/mL aprotinin, 1 mg/mL leupeptin, 5 mg/mL TLCK) and immunoprecipitated with anti-pygopus antisera overnight at 4°C. The primary antibody was bound to Protein A Sepharose beads. Immunoprecipitated protein was washed with Triton Media and 150 mM NaCl. Sample buffer was added and co-immunoprecipitated protein was detected by Western immunoblotting, as previously described.

2.7 Double labeling Immunocytochemistry (ICC)

2 x 10^4 TOV-112D, TOV-21G, IOSE 397, or NIH-OVCAR-3 cells were seeded in eight-well Lab-Tek® chamber slides (Nalge Nunc International, USA) sixteen hours prior to fixation. Cells were washed with cell culture PBS and fixed with 4% paraformaldehyde. Cells are treated with 0.2% Triton X-100 in ICC PBS, washed with
ICC PBS, and then blocked with 10% normal donkey serum (NDS). Antibodies for hPygo2 and β-catenin or hPygo2 with CENP-E were diluted in 1.5% NDS in ICC PBS and incubated with the cells in a humid chamber overnight at 4°C. Cells were again treated with 0.2% Triton X-100/ICC PBS and washed with ICC PBS. The biotinylated donkey anti-rabbit and cy3 donkey anti-mouse secondary antibodies were diluted in 1.5% NDS in ICC PBS and incubated with the cells for 30 minutes at room temperature. After the secondary antibody incubation, cells were washed, and strepavidin-FITC was conjugated to the biotin molecules. Fluorescent cells were viewed under a Olympus® BX50WI confocal microscope and photographs were taken using the Olympus® Fluoview FV3000 imaging software.

For CENP-E and hPygo2 double immunocytochemical staining, TOV-112D cells were synchronized with 2 mM thymidine and then immunocytochemically stained using hPygo2 and CENP-E antibodies. TOV-112D cells were first synchronized to have the maximum number of cells undergoing mitosis simultaneously. 4 x 10^4 TOV-112D cells were seeded in 4-well chamber slides. One day later they were treated with 2 mM thymidine. 36 hours later the thymidine was removed and the cells were washed with PBS and fresh DMEM was added. Eight hours later the cells were fixed, stained and viewed.

2.8 Antisense Knockdowns

Antisense oligonucleotides or siRNA was used to knockdown pygopus and β-catenin protein expression with the TOV-112D and TOV-21G cell lines. Both strategies were employed in each cell line with the most effective strategy presented in this thesis.
An antisense phosphorothioated oligonucleotide (5'-GGCTGAGCAAATCGTTOG0-3') was complimentary to the intermediary coding region (nucleotides 635-654 of hPygopus2, GenBank number AF457208). Control oligonucleotide sequences were the β-catenin mismatch (G*C*C*TGAGCTAATCATT*G*G*T where * represents phosphothioate bonds) and the human non-specific Xenopus Pygopus2 (T*T*T*GCGCCGTTTCTT*C*T*C). The hPygopus2 siRNA sequence (CCAGCCUCUGGGUCAAAAAC; target nucleotides 401-420) was designed and purchased using the online Qiagen online ordering system. β-catenin and non-specific siRNA was purchased as a siRNA/siAB™ Assay Kit from Upstate Biotechnology.

4 x 10^4 TOV-112D or TOV-21G cells were seeded in 24-well plates 16 hours prior to transfection. Using Oligofectamine Transfection Reagent (Invitrogen, USA), 250 nM of oligonucleotide was transfected into the TOV-21G cells. 0100 nM siRNA was transfected into the TOV-112D cell line using the RNAiFect™ transfection reagent (Qiagen). Media was changed 24 hours post-transfection. Cells were counted 48 and 72 hours post-transfection using a hemacytometer. To confirm the reduction in β-catenin and hPygo2 protein levels, Western immunoblots were performed as per the protocol described in Section 2.5.
Figure 2.3

hPygo2, β-catenin and BCL9 antisense oligonucleotide and siRNA sequences.
### Table: Name, Sequence, Target Location

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Target Location</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pygopus siRNA</strong></td>
<td>ACUGCAGCAGGAGUUUAU</td>
<td>2159-78</td>
</tr>
<tr>
<td><strong>Pygopus Oligo</strong></td>
<td>GGCTGAGCAAATCGTTGGG</td>
<td>635-54</td>
</tr>
<tr>
<td><strong>Non-specific Oligo</strong></td>
<td>T<em>T</em>T<em>GCGCCGTTTCTT</em>C<em>T</em>G</td>
<td>No complimentary sequence</td>
</tr>
<tr>
<td><strong>β-catenin siRNA</strong></td>
<td>ACUGCAGCAGGAGUUUAU</td>
<td>2159-78</td>
</tr>
<tr>
<td><strong>β-catenin siRNA</strong></td>
<td>Unavailable</td>
<td>Unspecified</td>
</tr>
<tr>
<td><strong>Non-specific siRNA</strong></td>
<td>Unavailable</td>
<td>No complimentary sequence</td>
</tr>
</tbody>
</table>

* = Phosphothioate Bond
CHAPTER 3
RESULTS

3.1 Pygopus localizes to the nucleus in EOC cell lines

I performed indirect double labeling immunocytochemistry to determine β-catenin and pygopus localization with the TOV-112D, TOV-21G, OVCAR-3, and IOSE 397 cells (see Figure 3.1). Nuclear accumulation of β-catenin is a hallmark of canonical Wnt pathway activation and was used to assess pathway activation status in these cell lines. Consistent with a β-catenin activating mutation, overexpressed β-catenin was found at high levels throughout TOV-112D cells, with pygopus co-localization occurring in the nucleus. Pygopus also localized in TOV-21G cells to the nuclei. For comparison, in NIH:OVCAR-3 cells β-catenin localized primarily to the membrane and pygopus was concentrated in the nucleus. The IOSE 397 cell line had perinuclearly-located pygopus and membrane-associated β-catenin. Because of its central role in Wnt-mediated transcription in other systems (Wright & Tijan, 2009; Mieszczanek et al, 2008; Nair et al, 2008; Fiedler et al, 2008; Jonckheere et al, 2008; reviewed in Jessen et al, 2008), I expected that if Pygopus was involved in Wnt signaling in these cells, it would be colocalized with β-catenin. On the contrary, the lack of β-catenin and pygopus nuclear co-localization within the TOV-21G cell line suggests that pygopus function in these cells is independent of β-catenin.

3.2 Endogenous Wnt activity is consistent with the presence of active β-catenin

We assayed TCF-dependent transcriptional activity to determine the presence and levels of endogenous canonical Wnt activity within the β-catenin-positive and -negative
Figure 3.1

Double immunocytochemistry of hPygo2 and β-catenin. iOSE397 immortalized epithelial ovarian cells, as well as TOV-112D, TOV-21G, and OVCAR-3 EOC cell lines were immunocytochemically stained using hPygo2 and β-catenin antibodies. Pre-immune sera was used as a control for the hPygo2 antibody. Scale bar equals 50 μm.

hPygo2 localized to the nucleus in the EOC cell lines and perinuclearly in the iOSE397 cells. β-catenin expression was variable, with it localizing to the cell membranes of iOSE397 and OVCAR-3 cells, expressed throughout the TOV-112D cells and is not detectable in the TOV-21G cells.
Chapter 3: Results

<table>
<thead>
<tr>
<th></th>
<th>hPygo2</th>
<th>hPygo2 + β-catenin</th>
<th>pre-immune + β-catenin</th>
</tr>
</thead>
<tbody>
<tr>
<td>iOSE397</td>
<td><img src="iOSE397_hPygo2.png" alt="Image" /></td>
<td><img src="iOSE397_hPygo2_%CE%B2-catenin.png" alt="Image" /></td>
<td><img src="iOSE397_pre-immune_%CE%B2-catenin.png" alt="Image" /></td>
</tr>
<tr>
<td>OVCAR-3</td>
<td><img src="OVCAR-3_hPygo2.png" alt="Image" /></td>
<td><img src="OVCAR-3_hPygo2_%CE%B2-catenin.png" alt="Image" /></td>
<td><img src="OVCAR-3_pre-immune_%CE%B2-catenin.png" alt="Image" /></td>
</tr>
<tr>
<td>TOV-21G</td>
<td><img src="TOV-21G_hPygo2.png" alt="Image" /></td>
<td><img src="TOV-21G_hPygo2_%CE%B2-catenin.png" alt="Image" /></td>
<td><img src="TOV-21G_pre-immune_%CE%B2-catenin.png" alt="Image" /></td>
</tr>
<tr>
<td>TOV-112D</td>
<td><img src="TOV-112D_hPygo2.png" alt="Image" /></td>
<td><img src="TOV-112D_hPygo2_%CE%B2-catenin.png" alt="Image" /></td>
<td><img src="TOV-112D_pre-immune_%CE%B2-catenin.png" alt="Image" /></td>
</tr>
</tbody>
</table>
Chapter 3: Results

Figure 3.2

Endogenous Wnt activity in Epithelial Ovarian Cancer cell lines. A. TCF-dependent transcriptional activity was determined by TOPflash assays. The presence and levels of endogenous canonical Wnt activity were determined in TOV-112D, TOV-21G, OVCAR-3, and SK-OV-3 cells. B. hPygo2 immunoprecipitation in TOV-112D and TOV-21G cells, followed by β-catenin Western blotting. β-catenin was found to bind with hPygo2 in the TOV-112D cells, but not in the TOV-21G cells. Total protein extracts are also shown.
Chapter 3: Results

A

Luciferase Activity (Fold Induction)

21G 112D OVCAR3 SKOV3

B

Total Protein  hPygo2 IP

TOV-112D TOV-21G TOV-112D TOV-21G

kDa 175 83 62  β-catenin
EOC cell lines. TOPflash vectors contain consensus TCF-binding sites located in sequence with a luciferase reporter gene. The TOPflash vector expresses luciferase when bound by the TCF-mediated canonical Wnt transcription complex. FOPflash plasmids are identical to TOPflash with the exception that they contain a mutated TCF binding site. Cells were transfected with either the wildtype TCF-dependent luciferase reporter vector (pTOPflash) or the mutated control vector (pFOPflash). The ratio of pTOPflash luciferase activity to FOPflash reporter activity is a measure of endogenous canonical Wnt activity. TOV-112D cells demonstrated a significant amount of constitutive canonical Wnt activity (see Figure 3.2) consistent with a known activating mutation in its \(\beta\)-catenin as demonstrated previously (Wu et al., 2001). On the other hand, in comparison, TOV-21G, OVCAR-3, and SKOV3 cells had baseline levels of canonical Wnt activity.

3.3 Pygopus transactivation activity is independent of \(\beta\)-catenin expression

The hPygopus2-Gal4 fusion constructs and pFR luc reporter plasmid were transfected into the TOV-112D and TOV-21G cell lines, as described in Figure 3.3A. When transfected, the hPygopus2-Gal4 fusion proteins were expected to bind the Gal4 binding site on the pFR luc plasmid and initiate expression of the luciferase reporter gene. The ability of the fusion proteins to activate transcription was measured by the level of luciferase enzyme activity. Figure 3.3B shows that within TOV-112D cells, all portions of pygopus showed significant transactivation activity with the PHD having the highest fold increase over baseline. Interestingly, the TOV-21G cells, which contain minimal amounts of \(\beta\)-catenin and have no discernable endogenous canonical Wnt
Figure 3.3

Gal4 Assays. A. Schematic representation of the Gal4 constructs provided by Phillip Andrews, Terry Fox Laboratory, Memorial University. Constructs include the Gal4 control (the empty pMG4 vector), αNHD + Δ (hPygo2 amino acids 1-312), PHD (amino acids 321-406), Δ (amino acids 74-312), NHDα (amino acids 1-95), and NHDβ (amino acids 38-95). B. The Gal4 assay performed in ovarian cancer cell lines TOV-112D and TOV-21G. 2 x 10^5 cultured cells were seeded in 6-well plates 24 hours prior to transfection. Using the Effectene® Transfection Reagent (Qiagen) 0.15 μg of each of the pFR luc reporter, a pM pygopus vector, and the RSV β-gal control plasmid were transfected into TOV-21G and TOV-112D cells. After 48 hours cells were washed with PBS and protein was extracted in Luciferase Cell Culture Lysis Buffer (Promega). Luciferase and β-galactosidase reporter expression was measured as previously described. Luciferase values were also normalized by total protein as determined by BioRad® assays. The experiment was performed in triplicate and repeated three times. C. Expression of the Gal4-hPygo2 constructs in TOV-21G and TOV-112D cells. The Western blot is representative. The experiment was performed in triplicate. Protein extracts were normalized and the amount loaded was calculated based on total protein.
concentration and β-actin Western immunoblot. D. Analysis of the transcription activation domain(s) in Pygo by Belenkaya et al., 2002 demonstrating hPygo2 was able to activate transcription. Cells were transfected with the pG5E1b-luciferase reporter construct (Hsu et al., 1994) and with vectors expressing GAL4 DNA-binding domain alone (pM1) (Sadowski et al., 1992) or with GAL4-Pygo fusion protein. A GAL4-Jun AC-containing Jun activation domain (amino acids 5 to 89) fused with GAL4 was used as a positive control. Luciferase activities are expressed as relative activities compared with cells transfected with the plasmid containing the GAL4 DNA-binding domain alone.
activity, also had significant amounts of transcriptional activitation from the pygopus fusion proteins. Although to a lesser extent, the PHD and Δ regions had the greatest fold increases.

3.4 Pygopus expression is required for the proliferation of EOC

To determine the requirements of Pygo and β-catenin, I reduced protein levels to approximately 50% of endogenous levels using antisense oligonucleotides in the TOV-21G cells and antisense siRNA in the TOV-112D cells (see Figures 3.4 and 3.5). The specificity of the protein knockdown was determined by Western immunoblotting. Both TOV-112D and TOV-21G EOC cell lines had significantly decreased proliferation in the hPygo-reduced samples as compared with the control mock transfected and non-specific siRNA and oligonucleotide transfected cells. This indicates that hPygo2 expression is required for the growth of these two EOC cell lines.

β-catenin protein levels were significantly decreased by antisense siRNA in the TOV-112D cell line. The reduction in β-catenin protein expression resulted in a decrease in TOV-112D proliferation as compared with the cells transfected with non-specific siRNA and mock-transfected cells. This reduction in proliferation was similar to the reduction in the hPygo2-reduced TOV-112D cells. β-catenin was not detectable in the TOV-21G cell line. Anti-β-catenin oligonucleotides did not decrease the proliferation of TOV-21G cells at either 48 or 72 hours post-transfection. BCL9 expression was reduced in the TOV-112D cells with BCL9 c and BCL9 d siRNA (see Figure 2.3 for a representation of the nucleotide changes). The reduction in proliferation of the TOV-112D cells treated with either the BCL9 c or BCL9 d siRNA was comparable to the
Figure 3.4

siRNA knockdown studies in TOV-112D EOC cells. A. Cell counts at 48 and 72 hours for TOV-112D cells transfected with nonspecific, hPygo2, β-catenin and BCL9 siRNA. Error bars represent standard error. B. Western analysis of hPygo2, β-catenin and, as a control, ERK performed 48 hours after knockdown with nonspecific, hPygo2, β-catenin and BCL9 siRNA. Results are based on three experiments performed in triplicate.
Chapter 3: Results

[Diagrams A and B are shown. Diagram A is a bar graph showing the total cell counts (thousands) at 48 and 72 hours after transfection with different siRNAs. Diagram B is a Western blot image showing protein bands for Mock, Non-specific, Pygopus, BCL9 c, BCL9 d, and beta-catenin siRNAs.]
Figure 3.5

Antisense oligonucleotide knockdown studies in TOV-21G EOC cells. A. Cell counts at 48 and 72 hours of TOV-21G cells transfected with nonspecific, hPygo2, β-catenin and BCL9 antisense oligonucleotides. B. Western analysis of hPygo2, β-catenin and, as a control, ERK 48 hours after knockdown with nonspecific, hPygo2, β-catenin and BCL9 antisense oligonucleotides. Results are based on three experiments performed in triplicate.
Chapter 3: Results

A

- Mock Transfected
- Non-specific oligo
- Mismatch oligo
- Antisense pygo oligo
- Antisense beta-catenin oligo

Total Cells (Thousands)

Hours after Transfection

B

- Transfected oligonucleotide
- pygopus
- β-catenin
- ERK
reduction seen in the TOV-112D cells treated with anti-hPygo2 or anti-β-catenin siRNA. No equivalent BCL9 antisense oligonucleotide was available for use with the TOV-21G cells. These results indicate that hPygo2 maybe acting differently in the Wnt-active TOV-112D cells than in the Wnt-inactive TOV-21G cells. hPygo2 is a known component of the Wnt transcription complex that includes β-catenin and BCL9. In the TOV-112D cells siRNA knockdowns of hPygo2, β-catenin and BCL9 produced similar decreases in proliferation, consistent with Wnt and the Wnt transcription complex playing an instrumental role in the proliferation of these cells. However in the Wnt-inactive TOV-21G cells, reductions in hPygo2 protein levels still resulted in decreased proliferation of the cells despite minimal Wnt activity, only faintly detectable expression of β-catenin and no significant effect of the anti-β-catenin oligonucleotide. This indicates that hPygo2 is required for the proliferation of the TOV-21G cells independently of β-catenin and the Wnt pathway.

3.5 Localization of hPygo2 in dividing TOV-112D cells

During my immunocytochemical analysis of the localization of hPygo2 within ovarian cancer cell lines, I observed discrete punctate areas of hPygo2 in cells that were dividing. It was hypothesized that Pygopus may have a role in mitosis. Cell cycle synchronization and double immunocytochemistry was performed with antibodies against Pygopus and the kinetochore protein, CENP-E. CENP-E associates with centromeres during congression where the chromosomes ultimately end up with their centromeres situated in middle of the spindle at the metaphase plate. It then relocates to the spindle midzone at anaphase, and is quantitatively discarded at the end of the cell division (Yen
et al, 1992). CENP-E is a centromere-associated protein and required for progression from metaphase to anaphase (Yen et al, 1991). The double-ICC I performed used CENP-E as a marker of mitosis, and showed discrete areas of hPygo2 staining during a period in the cell cycle where CENP-E staining was observed (see Figure 3.6). No CENP-E colocalization was observed indicating that these discrete punctuate areas of hPygo2 are not at the centromeres. However, using CENP-E as a marker of the metaphase to anaphase portion of the cell cycle, the immunocytochemical results indicate that Pygopus localizes to discrete punctuate areas during mitosis. Pygopus overexpression in the EOC cell lines I studied is consistent with it having a role in the uncontrolled mitosis that is occurring in these cells. Further study is needed to elucidate the non-Wnt functions of Pygopus.
Figure 3.6

Double immunocytochemistry of hPygo2 and CENP-E in synchronized TOV-112D cells. Thymidine-synchronized TOV-112D cells were immunocytochemically stained using hPygo2 and CENP-E antibodies.
Chapter 3: Results

<table>
<thead>
<tr>
<th>hPygo2</th>
<th>CENP E</th>
<th>hPygo2 + CENP E</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
</tr>
<tr>
<td><img src="image4.png" alt="Image" /></td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
<tr>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
<td><img src="image9.png" alt="Image" /></td>
</tr>
</tbody>
</table>
4.1 hPygo2 transactivation activity

TOV-112D cells demonstrated significant transactivation activity with the PHD and Δ having the highest fold increase over baseline. Interestingly, the TOV-21G cells, which contain minimal amounts of β-catenin and have no discernable endogenous canonical Wnt activity, also had significant amounts of transcriptional activation from the pygopus fusion proteins. Although to a lesser extent than in TOV-112D cells, the PHD and Δ regions had the greatest fold increase.

The activation with the PHD in TOV-112D cells may be partly explained by its ability to recruit the canonical Wnt transcription complex. A possible explanation for the Δ construct exhibiting transactivation activity is the recently discovered hPygo2 NPF motif. Städeli and Basler (2005) demonstrated that single point mutations of the uncharged amino acids N, P, or F at positions 76–78 resulted in an almost complete loss of transactivation activity in a UAS-luc assay where the hPygo2 NHD was tethered to DNA with the G4DBD in S2 cells. These important amino acids were present in the Δ construct and possibly provide a portion of its transactivation activity. However they are also present in the two NHD constructs, which have lower transactivation activities, and therefore the NPF motif cannot account for all the transactivation occurring within the Δ construct.

As there is no significant Wnt activity and minimal levels of β-catenin expression in TOV-21G cells, the transactivation activity of pygopus in TOV-21G cells is independent of β-catenin expression. However, β-catenin and the Wnt transcription
complex may be required for hPygo2 localization and DNA tethering. As the Gal4 transcription assay artificially tethers the hPygo2 construct to the transcription vector, the experiment only measures hPygo2’s ability to recruit and bind the necessary transcription machinery, but is not able to comment on its ability to bind the DNA promoter sequences.

4.2 **Wnt signaling and cancer**

Deregulation of the Wnt pathway leading to abnormal overexpression of mitogenic Wnt-target genes has been linked to many cancers (Bienz & Clevers, 2000; Polakis, 2000). 80% of all colorectal cancers show inactivation of APC which drastically reduces the phosphorylation and degradation of β-catenin. In the remaining 20%, many have activating point mutations in β-catenin that alter its GSK-3β phosphorylation sites causing a constitutive increase in its stability. Similar mutations in other Wnt components also result in β-catenin accumulation and ligand-independent target gene over-expression. Wnt components have been implicated in a number of cancers and diseases (see Table 1.3 and Section 1.2.4). There is much evidence, however, that the canonical Wnt pathway does not play a role in non-endometrioid EOC (Kildal *et al*, 2005; Faleiro-Rodrigues *et al*, 2004; Faleiro-Rodrigues *et al*, 2005, Popadiuk *et al.*, 2006).

My results are consistent with canonical Wnt signaling having a role in the proliferation of endometrioid EOC. TOV-112D cells were isolated from a 42 year old woman with a primary malignant adenocarcinoma of the ovary, specifically an endometrioid carcinoma, grade 3, stage IIIC (Provencher *et al*, 2000). In assays for TCF-dependent transcriptional activity, TOV-112D cells demonstrated a significant amount of
constituitive canonical Wnt activity (see Figure 3.2) consistent with a known activating mutation in β-catenin as demonstrated previously (Wu et al., 2001). As well, protein knockdowns experiments with anti-β-catenin and anti-BCL9 siRNA resulted in decreased proliferation of the TOV-112D cells.

4.3 Wnt-independent functions of pygopus

Pygopus is a component of the Wnt transcription complex. It associates with Armadillo/β-catenin and T cell factor (TCF) through the Legless/BCL9 adaptor, but its molecular function in TCF-mediated transcription is not completely described. Pygopus has been suggested to have a transactivation function (Kramps et al., 2002). For example, when fused to Gal4, Pygopus was able to activate transcription from a reporter vector containing a Gal4 binding site (Belenkaya et al., 2002). When overexpressed in Xenopus embryos, hPygo2 activated expression of the Wnt marker Engrailed-2 and induced secondary axis formation, two results consistent with Wnt activation (Lake and Kao, 2003). When fused to TCF, the pygopus NHD was able to activate TOPflash reporter expression (Thompson, 2004). This evidence suggests that pygopus acts as a transcriptional activator with the transactivation function dependent on the NHD.

Mieszczanek et al (2008) used a groucho-null allele to show that Groucho represses Wingless target genes during Drosophila development. Interestingly, groucho pygo double-mutants revealed that Pygo is not obligatory for transcriptional and phenotypic Wingless signaling in the absence of Groucho and other transcriptional antagonists of Wingless signaling. This indicated an anti-repressor function of Pygo.
The authors proposed that Pygo predisposes Drosophila TCF target genes for rapid Wingless-induced transcription or that it protects them against premature shut-down.

In the research that I have presented this thesis, I demonstrated that pygopus is required for proliferation of ovarian cancer cells both in the presence and absence of Wnt signaling. It is not unique, as other components of the Wnt pathway, such as β-catenin, axin, and APC, are engaged in cellular functions outside of canonical Wnt signaling (Bienz, 2002; Ciani et al., 2004). Previous research in our laboratory demonstrated that Pygopus was overexpressed in EOC (Popadiuk et al., 2006), despite the fact that there is much evidence that the canonical Wnt pathway does not play a role in non-endometrioid EOC (Kildal et al., 2005; Faleiro-Rodrigues et al., 2004; Faleiro-Rodrigues et al., 2005). As well, the Pygopus knockdown studies I performed demonstrated that pygopus was required for proliferation in both the Wnt-active TOV-112D and Wnt-inactive TOV-21G cell lines, indicating non-canonical Wnt activity for pygopus.

I went on to study one possibility of a non-canonical function of Pygopus. During my immunocytochemical analysis of the localization of Pygopus within ovarian cancer cell lines, I observed discrete punctate areas of Pygopus in cells that were dividing. It was hypothesized that Pygopus may have a role in mitosis. Cell cycle synchronization and double immunocytochemistry was performed with antibodies against Pygopus and the kinetochore protein, CENP-E. CENP-E associates with centromeres during congression, where the chromosomes ultimately end up with their centromeres situated in middle of the spindle at the metaphase plate. It then relocates to the spindle midzone at anaphase, and is quantitatively discarded at the end of the cell division (Yen et al., 1992). CENP-E is centromere-associated protein and required for progression from metaphase to
anaphase (Yen et al, 1991). The ICC I performed showed punctate staining of Pygopus on or around the CENP-E staining (see Figure 3.6). Using CENP-E as a marker of mitosis, the ICC staining indicates that Pygopus localizes to discrete areas during mitosis and may play a role in the cell cycle. Pygopus overexpression in the EOC cell lines I studied is consistent with it having a role in the uncontrolled mitosis that is occurring in these cells. Further study is needed to elucidate the non-Wnt functions of Pygopus.

4.4 Pygopus as a novel therapeutic target

With the prognosis of EOC remaining poor despite aggression surgical cytoreduction and chemotherapy, it is important to identify novel markers and therapeutic targets (See et al, 2003). A variety of mRNAs are now being used as targets for antisense therapy in early phase clinical trials (Wang et al, 2008; Xu et al, 2008; Gleave & Monia, 2005; See & Kavanagh, 2004), including vascular endothelial growth factor (Levine et al, 2006), protein kinase C alpha (Advani et al, 2004; Yuen et al, 1999), c-raf kinase (Oza et al, 2003; Cunningham et al, 2000), raf-1 (Mullen et al, 2004), p53 (Skilling et al, 1996), survivin (Ma et al, 2005), X-linked inhibitor of apoptosis protein (Li et al, 2000), and telomerase (Yuan et al, 2002; Kushner et al, 2002). The overexpression of hPygo2 in epithelial ovarian cancer cell lines and tumors (Popadiuk et al, 2006) and the requirement of hPygo2 for EOC growth that I confirmed in this thesis, strongly suggests that hPygo2 is a potential therapeutic target.

Pygopus shows more potential as a therapeutic target than other components of Wnt signaling. The Pygopus knockdown studies I performed demonstrate that Pygopus was required for proliferation in both the Wnt-active TOV-112D and Wnt-inactive TOV-
21G cell lines. Canonical Wnt is not consistently active in ovarian cancer cell lines, but pygopus is consistently overexpressed in EOC histologic subtypes (Popadiuk et al., 2006). Whereas canonical Wnt signaling appears to play a role in only endometrioid EOC, my work indicates hPygo2 is required for both endometrioid (TOV-112D) and non-endometrioid (TOV-21G) EOC cells. The work I have performed along with the previous work described, indicate hPygo2 has greater potential as a therapeutic target in EOC than other Wnt components, as the Wnt pathway is not universally required for Epithelial Ovarian Cancer proliferation.

hPygo2 is overexpressed in ovarian cancer cells, but in few other tissues. This specificity to EOC would limit the effect of hPygo2 antisense therapy on other cell types. More study is needed, but hPygo2 has potential for being a unique therapeutic target for a variety of EOC histologic subtypes with potentially less effect on other tissues due to its lack of expression.

Further research in our laboratory has shown that hPygo2 is required for anchorage-independent growth and growth in severe combined immunodeficient mice (Popadiuk et al., 2006). hPygo2 antisense oligonucleotides reduced anchorage-independent growth. There was a significant decrease in the size and number of colonies grown in soft agar formed by SK-OV-3 cells transfected with the pooled hPygo2 antisense oligonucleotides, compared with those transfected with the mismatched control oligo. The in vitro growth inhibition findings were complemented with in vivo growth assays to determine whether the in vivo culture environment might reverse the initial effects of hPygo2 antisense oligonucleotides. hPygo2 antisense oligonucleotides or mismatched control oligos were transiently transfected into SK-OV-3 cells and then
implanted subcutaneously into severe combined immunodeficient mice. Measurable
tumors appeared later in the mice implanted with the hPygo2 antisense oligonucleotide-
treated cells as compared with the missense-oligonucleotide transfected cells (11 weeks
versus 14 weeks). In all cases, antisense oligonucleotide–transfected cells formed smaller
tumors than mismatched control transfected cells and after 18 weeks, there was a visible
difference in tumor mass between mice implanted with cells transfected with hPygo2
antisense oligonucleotides (0.21 g) and those implanted with cells transfected with the
mismatched control oligos (0.62 g). These results indicated that in vivo culture of
hPygo2-transfected cells does not reverse the effect of growth inhibition by antisense
hPygo2 antisense oligonucleotides.

hPygo2 may also be a potential therapeutic target in other cancers. Andrews et al
(2007) demonstrated that hPygo expression was required for proliferation in breast cancer
cells. hPygo2 was highly expressed in breast cancer cell lines. The expression of
hPygo2 mRNA was highest in the malignant breast cell lines and showed a lower
expression level in normal breast cell lines (Hs-574, Hs-578-Bst). On the other hand, the
expression of hPygo1 mRNA was not specifically overexpressed in the malignant
cell lines and showed little correlation with the expression of hPygo2.

Immunohistochemical analysis showed hPygo2 localized to the nuclei of 64% of the
malignant tumours tested. While hPygo2 was absent from normal connective (stroma)
tissue and adipose tissue of the breast, it did accumulate weakly but asymmetrically in the
cytoplasm of ductal epithelium. In addition hPygo2, but not hPygo1, knockdowns in
MCF-7 cells and MDA-MB-468 cells produced a reduction in cell number and a decrease
in the Wnt target gene Cyclin D1. As an instrumental component of the Wnt
transcription complex and with minimal expression in normal breast tissue, hPygo2 has potential as a therapeutic target in breast malignancies.

Deregulation of Wnt signalling is linked to colorectal cancer (Polakis, 2000; Bienz and Clevers, 2000). 80% of all colorectal cancers show inactivation of APC. Furthermore, many colorectal tumours that are wild type for APC have activating mutations in β-catenin. Typically, they are point mutations that affect its GSK3 phosphorylation site, rendering β-catenin refractory to degradation. Colorectal cancer cells that are mutant for APC or β-catenin have high levels of TCF-mediated transcription (Morin et al, 1997), which is thought to be the basis for tumorigenesis.

Thompson et al (2002) used double-stranded (ds) RNA interference on colorectal cancer cells that have high levels of β-catenin–TCF-mediated transcription, to test the function and requirement of endogenous Pygo proteins. This transcriptional activity was measured with a luciferase reporter (TOPFLASH) that contains multiple TCF binding sites. TOPFLASH activity was high in colorectal cancer cells that are mutant for APC (for example, SW480 cells) or that have an oncogenic β-catenin mutation (for example, HCT116 cells). They designed RNA oligomers specific for β-catenin, hPygo1 and hPygo2, and co-transfected SW480 and HCT116 cells with the ds RNAi oligomers, TOPFLASH and an internal reference plasmid. They found that in both the SW480 and HCT116 cells, the β-catenin, hPygo1 and hPygo2 oligomers all caused a substantial reduction of TOPFLASH activity, to 35% of controls (Fig. 6g,h). hPygo2 oligomers are less effective; they roughly halve TOPFLASH activity (Fig. 6g,h). The combined application of both oligomers, simultaneously or in succession, results in reductions to 35–40% of controls (Fig. 6h and data not shown). There are no specific effects of the
Pygo oligomers on FOPFLASH activity (Fig. 6h, middle). Therefore, the effects of the Pygo oligomers on the TOPFLASH activity are significant and specific. Finally, cotransfection of cells with mPygo1 rescues the effects of both hPygo2 and hPygo1 oligomers, but does not rescue the effects of β-catenin depletion. They concluded that both human Pygo proteins are required for Wnt-dependent TCF-mediated transcription in colorectal cancer cells. While not assayed, growth of these cells is predicted to be inhibited by Pygo knockdown consistent with that shown for β-Catenin (Roh et al, 2001; Verma et al, 2003; Sekiya et al, 2002).

hPygo2 may also play a role in chemotherapeutic resistance. In a recent study, De et al (2009) generated human carcinoma (HeLa) cell lines stably expressing Pygo2, which counteracted vinblastine-induced apoptosis. The anti-apoptotic function was determined by various techniques including DNA fragmentation, sub-G1 appearance, loss of mitochondrial membrane potential (Deltapsim) and the activation of caspase-9 and caspase-3. In addition, they found that Pygo2 effectively blocks vinblastine-induced c-Jun and AP-1 activation, maintains the anti-apoptotic protein Bcl-2 in an unphosphorylated state, and therefore rendered cells resistant to apoptosis. They concluded the anti-apoptotic activity exerted by Pygo2 was through blocking activation of the JNK/AP-1 signaling pathway induced by vinblastine. This chemotherapeutic resistance is the first to be linked to hPygo2 and more research in this area is sure to follow.

4.5 Conclusions

In conclusion I have confirmed the hypothesis that hPygo2 overexpression
contributes to the growth of ovarian cancer cells. I have shown this through hPygo2 expresional knockdown experiments where hPygo2-knocked-down ovarian cancer cells had decreased proliferation. Decreased hPygo2 levels decreased ovarian cancer cell proliferation independent of Wnt activity, as seen in the Wnt-inactive TOV-21G cells. This indicates that hPygo2 would make a more suitable therapeutic target than other Wnt proteins. More research into hPygo2's Wnt-independent and Wnt-dependent functions is required to understand its role in ovarian cancer proliferation.

4.6 Future directions

My results indicate that hPygo2 is involved in the pathogenesis of ovarian cancer. A study with a large patient population would be needed to confirm this. Prognosis, disease-free survival, and response to treatment could be correlated to hPygo2 expression to determine if it is a valid prognostic marker.

There has been little work done on the Wnt-independent functions of hPygo2. As my results confirm that hPygo2 is important in ovarian cancer cell proliferation independent of Wnt activity, it is important to determine the functions that are important in cancer pathogenesis. Proteomics is a potential tool that can be used to further elucidate the role of hPygo2, both within the Wnt transcription complex and in the absence of Wnt. hPygo2 and its interacting proteins could be immunoprecipitated using the antibody developed within our laboratory. Candidate proteins would then have to be identified, confirmed to interact with hPygo2 and then evaluated for their functional significance.

After hPygo2 immunoprecipitation, interacting proteins could be identified by a couple techniques. First interacting proteins would be separated by a high resolution,
two-dimensional gel electrophoresis. Protein bands could be viewed by a non-specific stain such as Coomassie Blue. The different banding patterns between the Wnt-active (TOV-112D) and Wnt-inactive (TOV-21G) cells would be indicative of proteins that bind to hPygo2 in the presence and absence of the Wnt pathway. Bands present in the TOV-21G, but not TOV-112D cell precipitates would represent proteins that are possibly important for hPygo2's Wnt-independent functions. Similarly, bands present in the TOV-112D, but not the TOV-21G immuno precipitates would possibly represent proteins important for hPygo2's functions in the Wnt pathway. The bands could be cut out of the gel, purified and analysed by matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry. MALDI is a soft ionization technique used to analyze biomolecules that are otherwise too fragile to be ionized by conventional techniques. Commonly a pulsed nitrogen laser is used to initiate the ionization. The laser is first passed through a crystallized matrix that protects the molecule from being destroyed as would be the case with direct laser ionization. The TOF mass spectrometer accelerates the ionized particles using an electromagnetic field. The velocity of the ion depends on the mass-to-charge ratio. The time that it subsequently takes for the particle to reach a detector is measured. This time will depend on the mass-to-charge ratio of the particle (heavier particles reach lower speeds). From this time one can find the mass-to-charge ratio of the ion which can be compared to a database to identify the protein.

An alternative to MALDI-TOF is Tandem mass Spectrometry, also known as MS/MS (Hardouin, 2007; Shadforth et al, 2005; Mørtz et al, 1996). A tandem mass spectrometer can be thought of as two mass spectrometers in series connected by a collision cell. A protein sample is sorted in the first mass spectrometer, broken into
smaller peptides in the collision cell, and a single peptide or a small number of peptides are sorted and analyzed in the second mass spectrometer. The mass spectrometers can be a sector MS that utilizes a static electric or magnetic sector or some combination of the two as a mass analyzer, a transmission quadrupole MS that separates ions based on the stability of their trajectories in oscillating electric fields applied to four parallel circular rods, or time-of-flight as discussed above. The first MS would select out proteins and peptides of interest. The second MS could identify the amino acid sequences of those peptides that could be then compared to genome sequences to identify previously unknown proteins that interact with hPygo2. Tandem MS is useful in samples that contain dozens or hundreds of compounds as the first MS will allow for the analysis of a just a few by the second MS. It may not be practical to identify every compound in the samples, so this would allow for the analysis of a smaller number of proteins of interest that may have significance. It would eliminate some of the purification steps after the initial immunoprecipitation and the possible loss of important proteins.
CHAPTER 5
REFERENCES


Albers DS; Marth C; Alvarez RD; Johnson G; Bidzinski M; Kardatzke DR; Bradford WZ; Loutit J; Kirn DH; Clouser MC; Markman M (2008). Randomized phase 3 trial of interferon gamma-1b plus standard carboplatin/paclitaxel versus carboplatin/paclitaxel


Bookman, MA for the Gynecologic Cancer InterGroup (GCIG) (2006). GOG0182-ICON5: 5-arm phase III randomized trial of paclitaxel (P) and carboplatin (C) vs combinations with gemcitabine (G), PEG-liposomal doxorubicin (D), or topotecan (T) in patients (pts) with advanced-stage epithelial ovarian (EOC) or primary peritoneal (PPC) carcinoma. Journal of Clinical Oncology, 2006 ASCO Annual Meeting Proceedings Part I. 24(18S): 5002

Bosetti C; Negri E; Franceschi S; Pelucchi C; Talamini R; Montella M; Conti E; La Vecchia C (2001). Diet and ovarian cancer risk: a case-control study in Italy. Int J Cancer 93(6):911-5.


Chang ET; Lee VS; Canchola AJ; Clarke CA; Purdie DM; Reynolds P; Anton-Culver H; Bernstein L; Deapen D; Peel D; Pinder R; Ross RK; Stram DO; West DW; Wright W; Ziogas A; Horn-Ross PL (2007). Diet and risk of ovarian cancer in the California Teachers Study cohort. *Am J Epidemiol.* 165(7):802-13.


du Bois A; Weber B; Rochon J; Meier W; Goupil A; Olbricht S; Barats JC; Kuhn W; Orfeuvre H; Wagner U; Richter B; Lueck HJ; Pfisterer J; Costa S; Schroeder W; Kimmig R; Pujade-Lauraine E (2006). Addition of epirubicin as a third drug to carboplatin-paclitaxel in first-line treatment of advanced ovarian cancer: a prospectively randomized gynecologic cancer intergroup trial by the Arbeitsgemeinschaft Gynaekologische Onkologie Ovarian Cancer Study Group and the Groupe d'Investigateurs Nationaux pour l'Etude des Cancers Ovariens. J Clin Oncol. 24(7):1127-35.


Appendices Wells; page 131


Kohn EC; Sarosy G; Bicher A; Link C; Christian M; Steinberg SM; Rothenberg M; Adamo DO; Davis P; Ognibene FP; et al (1994). Dose-intensive taxol: high response rate in patients with platinum-resistant recurrent ovarian cancer. J Natl Cancer Inst 86(1): 18-24.


Koushik A; Hunter DJ; Spiegelman D; Anderson KE; Buring JE; Freudenheim JL; Goldbohm RA; Hankinson SE; Larsson SC; Leitzmann M; Marshall JR; McCullough ML; Miller AB; Rodriguez C; Rohan TE; Ross JA; Schatzkin A; Schouten LJ; Willett WC; Wolk A; Zhang SM; Smith-Warner SA (2006). Intake of the major carotenoids and the risk of epithelial ovarian cancer in a pooled analysis of 10 cohort studies. Int J Cancer. 119(9):2148-54.


Kramps, T., Peter, O., Brunner, E., Nellen, D., Froesch, B., Chatterjee, S., Murone, M., Züllig, S., & Basker, K. (2002). Wnt/Wingless signaling requires BCL-9/Legless-


Appendices


Appendices


Appendices


Piccart MJ; Bertelsen K; James K; Cassidy J; Mangioni C; Simonsen E; Stuart G; Kaye S; Vergote I; Blom R; Grimshaw R; Atkinson RJ; Swenerton KD; Trope C; Nardi M; Kaern J; Tumolo S; Timmers P; Roy JA; Lhoas F; Lindvall B; Bacon M; Birt A; Andersen JE; Zee B; Paul J; Baron B; Pecorelli S (2000). Randomized intergroup trial of cisplatin-paclitaxel versus cisplatin-cyclophosphamide in women with advanced epithelial ovarian cancer: three-year results. *J Natl Cancer Inst* **92**(9):699-708.


Prentice RL; Thomson CA; Caan B; Hubbell FA; Anderson GL; Beresford SA; Pettinger M; Lane DS; Lessin L; Yasmeen S; Singh B; Khandekar J; Shikany JM; Satterfield S; Chlebowski RT (2007). Low-fat dietary pattern and cancer incidence in the Women's Health Initiative Dietary Modification Randomized Controlled Trial. *J Natl Cancer Inst.* **99**(20):1534-43.


Qin M, Hayashi H, Oshima K, Tahira T, Hayashi K, Kondo H (2005). Complexity of the genotype-phenotype correlation in familial exudative vitreoretinopathy with mutations in the *LRP5* and/or *FZD4* genes. *Hum Mutat* **26**:104–112


Appendices


Steevens J; Schouten LJ; Verhage BA; Goldbohm RA; van den Brandt PA (2007). Tea and coffee drinking and ovarian cancer risk: results from the Netherlands Cohort Study and a meta-analysis. *Br J Cancer.* 97(9):1291-4.


The Wnt Gene Hompage. Website: http://www.stanford.edu/~russe/wntwindow.html


