Novel Roles for 185delAG Mutant BRCA1

in Ovarian Cancer Pathology

by

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of the requirements for the degree of
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Dedication

I dedicate this dissertation to my wonderful husband, who has been a bottomless well of patience, strength, encouragement, and love throughout its completion. I love you. I would also like to thank my parents, who have supported my every dream since they first taught me how to have them, and have believed in me every step of the way. I would like to thank Dr. Kruk, my mentor, for accepting me into her lab and giving me the opportunity to continue my education at USF. Thank you for teaching me to be a better scientist and writer than I ever could have been without you. Lastly, I would like to thank my lab mates, Nicole, Christina, and Kamisha, for keeping me going and keeping me laughing even on the worst days. I could not have survived this journey without you.
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# Table of Contents

List of Tables ............................................................................................................................ iv

List of Figures ................................................................................................................................v

List of Abbreviations .................................................................................................................. vii

Abstract........................................................................................................................................ xi

Chapter 1: Background .............................................................................................................. 1
  Ovarian Cancer .......................................................................................................................... 1
    Clinical characteristics of ovarian cancer ................................................................................ 1
    Origins of ovarian cancer ........................................................................................................ 2
    Models of ovarian cancer ....................................................................................................... 6
    Risk Factors for ovarian cancer development ......................................................................... 7
  BRCA1 ........................................................................................................................................ 7
    Introduction ............................................................................................................................ 7
    The BRCA1 gene product ....................................................................................................... 8
    Types of BRCA1 mutations ................................................................................................. 9
    Loss of function mutations ............................................................................................... 11
    Gain of function mutations ............................................................................................... 14
    The role of gain of function mutations for development, cellular
      proliferation, chemosensitivity, apoptosis, and gene regulation ........................................... 16
    Clinical impact of gain of function mutations ..................................................................... 20
  MMP1 ....................................................................................................................................... 22
    Introduction ............................................................................................................................ 22
    Structure and function ......................................................................................................... 23
    Regulation ............................................................................................................................. 26
    MMPs and cancer .................................................................................................................. 28
    Rationale ............................................................................................................................... 30
  Central Hypothesis .................................................................................................................. 33
  Specific Aims ............................................................................................................................ 33
  References ................................................................................................................................ 34

Chapter 2: The 185delAG BRCA1 Mutation Enhances MMP1 Expression in
  Human Ovarian Surface Epithelial Cells .................................................................................. 47
  Introduction ............................................................................................................................... 47
  Methods ..................................................................................................................................... 49
    Cell culture and transfection ............................................................................................... 49
Results

BRAT alters expression of genes involved in multiple cellular processes ...........................................54
BRAT enhances MMP1 gene expression in HOSE 118 cells ..................55
BRAT increases expression and secretion of pro-MMP1 by HOSE 118 cells ..................................57
MMP1 and maspin are independent targets of BRAT .........................57
BRAT-mediated MMP1 modulation is c-Jun dependent ......................61
AP-1 sites in the MMP1 promoter mediate enhanced MMP1 expression in BRAT cells ..........................65
Increased pro-MMP is detectable in BRAT mutation carrier-derived cellular conditioned media ........................................67

Discussion ..........................................................................................................................70

References .........................................................................................................................81

Chapter 3: Impact of BRAT on Apoptosis, Gene Regulation, and Migration of Human Breast Cancer Cells ..........87

Introduction ..........................................................................................................................87

Methods .............................................................................................................................90

Cell culture and transfection ..............................................................................................90
Cell viability assay .............................................................................................................91
Western blot .....................................................................................................................91
RT-PCR ............................................................................................................................91
Scrape assay .....................................................................................................................92
Statistics ............................................................................................................................93

Results .............................................................................................................................93

Endogenous BRCA1 and exogenous BRAT expression levels in normal breast epithelial and breast cancer cells ..........93
BRAT does not significantly impact proliferation or chemosensitivity of normal breast or breast cancer cells ...........94
BRAT does not significantly impact maspin expression in normal breast epithelial or breast cancer cells ..................101
BRAT does not significantly alter MMP1 expression levels in normal breast epithelial or human breast cancer cells .........................................................102
BRAT does not significantly impact migration of breast cancer cells ..................................................................105

Discussion ..........................................................................................................................105

References .........................................................................................................................113

Chapter 4: Conclusions .....................................................................................................118
List of Tables

Table 1.1. Studies supporting loss or gain of function mutation as mechanisms of enhanced BC and OC risk ................................................................. 12

Table 2.1. Selection of genes determined to be differentially regulated in BRAT cells ........................................................................................................... 56
List of Figures

Figure 1.1. BRCA1 mutations and their cellular and physiologic impact ......................10

Figure 1.2. MMP1 domain structure and potential substrates of importance in OC ..........24

Figure 1.3. Maspin in part mediates the enhanced apoptotic response of BRAT cells to STS treatment .........................................................................................................................32

Figure 2.1. MMP1 mRNA is increased in BRAT-expressing HOSE cells .....................58

Figure 2.2. BRAT increases cellular pro-MMP1 and total secreted MMP1 in BRAT cells ........................................................................................................................................................59

Figure 2.3. MMP1 and maspin are parallel targets of BRAT .........................................62

Figure 2.4. BRAT-mediated MMP1 modulation is c-Jun dependent ...............................63

Figure 2.5. AP-1 sites in the MMP1 promoter mediate enhanced MMP1 expression in BRAT cells ..................................................................................................................................................66

Figure 2.6. Increased pro-MMP1 is detectable in BRAT mutation carrier-derived cellular conditioned media ..........................................................................................................................71

Figure 2.7. ETs-1 protein levels are elevated in BRAT cells ............................................74

Figure 2.8. Constitutively active Akt reverses BRAT-mediated MMP1 up-regulation .........................................................................................................................................................76

Figure 3.1. Wild type BRCA1 levels in normal human breast epithelial and breast cancer cells ........................................................................................................................................95

Figure 3.2. Transfection efficiency of normal breast epithelial and breast cancer cell lines ....................................................................................................................................................96

Figure 3.3. BRAT is efficiently expressed in SKBr3 cells .................................................97

Figure 3.4. BRAT does not significantly impact growth or chemosensitivity of breast cancer cells ...............................................................................................................................................99
Figure 3.5. BRAT does not significantly impact maspin expression in breast cancer cells. ..............................................................103

Figure 3.6. MMP1 expression is not altered by BRAT in normal breast epithelial or breast cancer cells. ..............................................................106

Figure 3.7. Migration of SKBr3 cells is not significantly impacted by BRAT ...............107

Figure 4.1. Pro-IL1 β levels are increased in conditioned media of BRAT cells ............121
**List of Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>α2M</td>
<td>α2 macroglobulin</td>
</tr>
<tr>
<td>(aa)</td>
<td>Amino acids</td>
</tr>
<tr>
<td>AKT1/PKB</td>
<td>Protein kinase B</td>
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<tr>
<td>AP1</td>
<td>Activator protein 1</td>
</tr>
<tr>
<td>APC</td>
<td>Adenomatous polyposis coli</td>
</tr>
<tr>
<td>APMA</td>
<td>4-Aminophenylmercuric acetate</td>
</tr>
<tr>
<td>BARD1</td>
<td>BRCA1-associated RING domain 1</td>
</tr>
<tr>
<td>BCL-2</td>
<td>B cell lymphoma 2</td>
</tr>
<tr>
<td>BCL-2A1</td>
<td>BCL-2 related protein A1</td>
</tr>
<tr>
<td>BRAT</td>
<td>BRCA1 185delAG Amino Terminal truncated protein</td>
</tr>
<tr>
<td>BRCA1</td>
<td>Breast and Ovarian Cancer Susceptibility Gene 1</td>
</tr>
<tr>
<td>BRCT</td>
<td>BRCA1 C-terminal</td>
</tr>
<tr>
<td>CA-125</td>
<td>Cancer antigen 125</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>cIAP1</td>
<td>Cellular inhibitor of apoptosis 1</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element binding</td>
</tr>
<tr>
<td>Ct</td>
<td>Threshold cycle</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence substrate</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
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EGFR  Epidermal growth factor receptor
ELISA  Enzyme-linked immunosorbant assay
EMMPRIN  Extracellular Matrix Metalloprotease Inducer
ERK  Extracellular signal regulated kinase
ETS-1  v-ets erythroblastosis virus E26 oncogene homolog 1
FBS  Fetal bovine serum
FOL1  Folate receptor alpha
GFP  Green fluorescent protein
h  Hours
Her2  Human epidermal growth factor receptor 2
HGF  Hepatocyte growth factor
HOSE  Human ovarian surface epithelium
HRP  Horseradish peroxidase
HUVEC  Human umbilical vein endothelial cell
IGF-1  Insulin-like growth factor 1
IGF-1R  Insulin-like growth factor 1 receptor
IL-1  Interleukin-1
JNK/SAPK  c-Jun N-terminal kinase/stress activated protein kinase
kDA  Kilodalton
kRAS  Kirsten rat sarcoma viral oncogene homolog
LATS1  Large tumor suppressor 1
LOH  Loss of heterozygosity
LPA  Lysophosphatidic acid
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Macrophage colony stimulating factor</td>
</tr>
<tr>
<td>MMP1</td>
<td>Matrix metalloprotease 1</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>MSH2</td>
<td>Human mutS homolog 2</td>
</tr>
<tr>
<td>NES</td>
<td>Nuclear export signal</td>
</tr>
<tr>
<td>NFKB2</td>
<td>Nuclear factor kappa B 2</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear localization signal</td>
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<tr>
<td>OC</td>
<td>Ovarian cancer</td>
</tr>
<tr>
<td>OVX1</td>
<td>OC cell line antigen</td>
</tr>
<tr>
<td>P21</td>
<td>Cyclin dependent kinase inhibitor protein 21</td>
</tr>
<tr>
<td>P300</td>
<td>E1A binding protein 300</td>
</tr>
<tr>
<td>P53</td>
<td>Tumor protein 53</td>
</tr>
<tr>
<td>PAR-1</td>
<td>Protease activated receptor 1</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>PEA3</td>
<td>Polyoma enhancer activator protein 3</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>RQ</td>
<td>Relative mean mRNA expression</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
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<tr>
<td>Acronym</td>
<td>Full Name</td>
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<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>STS</td>
<td>Staurosporine</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor α</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue inhibitor of metalloprotease</td>
</tr>
<tr>
<td>TPD52</td>
<td>Tumor protein D52</td>
</tr>
<tr>
<td>uPA</td>
<td>Urokinase plasminogen activator</td>
</tr>
<tr>
<td>uPAR</td>
<td>Urokinase plasminogen activator receptor</td>
</tr>
<tr>
<td>Wt</td>
<td>Wild type</td>
</tr>
<tr>
<td>XIAP</td>
<td>X-linked inhibitor of apoptosis protein</td>
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Abstract

Familial history is the strongest risk factor for developing ovarian cancer (OC), and a significant contributor to breast cancer risk. Most hereditary breast cancers and OCs are associated with mutation of the tumor suppressor Breast and Ovarian Cancer Susceptibility Gene 1 (BRCA1). Studying risk-associated BRCA1 truncation mutations, such as the founder mutation 185delAG, may reveal signaling pathways important in OC etiology. Recent studies have shown novel BRCA1 mutant functions that may contribute to breast and OC initiation and progression independent of the loss of wtBRCA1. Previously, we have found that normal human ovarian surface epithelial (HOSE) cells expressing the 185delAG mutant, BRAT (BRCA1 185delAG Amino Terminal truncated protein), exhibit enhanced chemosensitivity and up-regulation of the OC-associated serpin, maspin. In the current study, I identify an additional target of the BRAT mutation, matrix metalloprotease 1 (MMP1), a key player in invasion and metastasis. BRAT-expressing HOSE cells exhibit increased MMP1 messenger RNA (mRNA) by real time PCR and protein by Western blotting. Pro-MMP1 levels are also higher in conditioned media of BRAT-expressing cells and HOSE cell lines derived from BRAT mutation carriers. c-Jun is critical for BRAT-mediated MMP1 up-regulation, as siRNA knockdown diminishes MMP1 levels. Luciferase reporter constructs reveal that activator Protein 1 (AP1) sites throughout the distal end of the promoter contribute to BRAT-mediated MMP1
expression, and basal activity is mediated in part by an AP1 site at (-72).
Reporters containing a single nucleotide polymorphism (SNP) associated with OC risk and progression yield increased activity that is further enhanced in BRAT cells. Interestingly, BRAT-mediated changes in chemosensitivity and gene regulation are not recapitulated in a normal breast epithelial or breast cancer cell model. This suggests tissue-specific mutant BRCA1 functions may contribute to breast and ovarian tissue specificity of BRCA1 mutation-associated cancer risk and also to differential breast and ovarian cancer risk and penetrance associated with specific mutations. Early molecular and cellular changes such as MMP1 up-regulation in the ovarian surface epithelium of BRCA1 mutation carriers may promote OC initiation and progression and represent a step forward on the continuum of cellular malignancy. Further investigation is warranted, as elucidating these early changes will aid in identification of potential screening and treatment strategies.
Chapter 1:

Background

Ovarian Cancer

**Clinical characteristics of ovarian cancer.** According to the American Cancer Society’s 2010 statistics, ovarian cancer (OC) is the 9th most common cancer in women, but ranks 5th in cancer related deaths [1]. The deadliness of this disease can be attributed to multiple challenges, including lack of a conclusive screening method, the fact that early stages of OC are virtually asymptomatic, and lack of a clear molecular profile. These challenges have limited experimental and clinical research to little progress in increasing the survival rate for this deadly disease in the last 7 decades [1].

Because early stage OC is difficult to detect, much investigation has been done to find early OC markers for use in screening. Some potential markers under investigation include osteopontin, macrophage colony stimulating factor (M-CSF), OVX1 (OC cell line antigen), lysophosphatidic acid (LPA) [2], B cell lymphoma 2 (BCL-2) [3], angiostatin [4], and the current gold standard, cancer antigen 125 (CA-251) [2]. Analyzing the proteome signatures of OC patient serum has also been suggested as a screening tool, as well as a way to improve assignment of prognosis and treatment strategy [5]. Other screening methods include pelvic exam and trans-vaginal ultrasound [6], though so far, screening of this type does not significantly decrease mortality [2].
The clinical behavior of OC presents a formidable challenge to improving long

term survival. Metastasis plays a major role in this difficulty. The majority of OC
diagnoses are made in Stage 3 and 4, when the primary tumor has metastasized and
patient survival falls below 30% [1]. In contrast, stage 1 OCs, which are confined to the
ovary, have a survival rate approaching 95% [1]. Typically, late stage patients present
with symptoms of abdominal pain and distention because of accumulating fluid and
extensive tumor growth within the peritoneum. Cancer encompasses the ovaries, and
shed tumor cells are found in the intraperitoneal fluid (ascites). These cells disseminate
easily throughout the peritoneal cavity, and have frequently formed metastases in the
omentum and peritoneum, as well as through the peritoneum and into the stroma of other
organs [7]. First line treatment involves debulking surgery and chemotherapy. Sixty to
eighty percent of patients benefit significantly from systemic or intraperitoneal treatment
with a platinum-based drug, specifically carboplatin, which is often combined with a
taxane [8]. Ultimately, the majority of patients eventually develop resistance and
experience disease recurrence within 18 months [9]. Patients who progress further and
those who do not respond to first line platinum-based therapy with a remission greater
than 6 months may be treated with alternative drugs, such as Gemcitabine, Bevacizumab,
or Tamoxifen [10], though responses are uniformly poor. Despite these treatment efforts,
second line therapy is generally accepted as a palliative strategy.

Origins of ovarian cancer. To better detect and treat OC, much investigation has
sought the origins of OC. Ninety percent of OCs arise from the surface epithelium of the
ovary [11], while the remainder are categorized as sex cord-stromal tumors, germ cell
tumors, or indeterminate tumors [6]. Hereafter, OC will refer to epithelial ovarian cancer.
HOSE is a non-stratified squamous or cuboidal epithelium that exhibits apical microvilli, cilia, simple desmosomes, and incomplete tight junctions, and expresses keratins, integrins, and N-cadherin [11]. HOSE shares a common developmental progenitor with the mesothelial layer lining the peritoneal cavity. Both types of cells arise from the mesodermally-derived celomic epithelium, which lines the embryonic celomic cavity. Specifically, the celomic epithelium covering the paired gonadal ridges proliferates on the surface to form the HOSE. Some cells also migrate into the gonadal ridges through the stroma and differentiate to become granulosa cells [11]. The Mullerian ducts, which later develop into the oviducts, uterus, cervix, and upper vagina, also arise from the celomic epithelium [12].

The embryologic origin of HOSE is extremely important to understanding OC. In contrast to most cancer types, in which malignant cells become less differentiated as the tumor progresses, malignant HOSE cells acquire a more mature, more differentiated phenotype than normal HOSE, and resemble specific types of Mullerian epithelia. Serous tumors are the most common type of epithelial OC and are comprised of cystic, solid, and papillary regions [13]. The epithelium resembles that of the Fallopian tube, as cells are columnar and ciliated, and form papillae [11]. Mucinous tumors are characterized by stratified cervical or intestinal-like epithelial cells that express mucin and form cysts and glands [13]. Endometrioid tumors are solid tumors with fluid-filled cysts, and the cells resemble endometrial epithelium [13]. Clear cell OCs exhibit tubular, solid, papillary or mixed growth, and are comprised of cells full of clear cytoplasm and peg-shaped cells with apical nuclei [13].
The mesodermal origin of the HOSE is also important for understanding OC because it imparts upon HOSE cells a unique bi-potential epithelial-mesenchymal hybrid phenotype, which may contribute to the ability of HOSE cells to develop a malignant phenotype. For example, HOSE cells co-express the mesenchymal marker vimentin with epithelial keratins [11]. On some substrates in culture, HOSE cells become contractile [14], fibroblast-like, invasive, down-regulate expression of some integrins, and secrete proteases and basal lamina and stromal components such as laminin, and collagen I, III, and IV [15]. The potential of HOSE cells to exhibit mesenchymal characteristics such as these are likely important to their normal physiologic function of restoring the normal HOSE and extracellular matrix (ECM) structure after ovulatory rupture. This ability to adapt to and modify the ECM could impart an advantage to HOSE cells in invasion and metastases.

Though it is clear that the embryologic development of HOSE is important for OC initiation and progression, the early stages of OC are still largely unclear. Unlike some cancer types, such as colon cancer, a clear stage-by-stage progression from normal tissue to pre-cancerous neoplasia to malignant disease has not been defined. One theory is that OCs arise from the metaplastic epithelium of surface invaginations and inclusion cysts, which are epithelium-lined cysts within the ovarian stroma that have lost contact with the surface of the ovary. These regions increase in frequency with age. Much of the evidence supporting the theory that inclusion cysts and invaginations are the origin of OC comes from prophylactically removed normal ovaries from patients with a known OC-associated BRCA1 mutation or a strong family history of OC. In some studies, this tissue exhibits more invaginations in the ovary surface, dysplasia, hyperplasia, and/ or surface
papillae compared to normal ovaries [16-18], though contrary findings have been reported [19, 20]. Additionally, occlusion cysts and cytologic changes are more common in the unaffected ovary of OC patients, and occlusion cysts frequently express markers associated with malignant OC, such as CA-125, E-cadherin, and tumor protein 53 (p53) [11, 21-23].

An alternative hypothesis is that serous OCs actually originate from the Fallopian tube epithelium (Reviewed in [24]). The histologic similarity of tubal intraepithelial carcinomas to serous OC and frequency of extensive intraperitoneal tumor growth at diagnosis makes identification of primary tubal intraepithelial carcinoma difficult. Nonetheless, this hypothesis merits consideration, as several studies have reported genetic changes common to serous OC and putative tubal intraepithelial carcinoma in normal fallopian epithelium and benign regions of hyperplasia [25]. For example, in prophylactically removed fallopian tubes of BRCA1 mutation carriers, regions of p53 overexpression frequently coincide with and have been found adjacent to tubal intraepithelial carcinoma, suggesting that these regions may represent a pre-malignant precursor [26]. Further, in at least one study, primary fallopian tube carcinomas have been found more frequently in BRCA1 mutation carriers [27]. Further study will prove valuable in determining the contribution of tubal intraepithelium to serous OC in these patients.

Finally, in contrast to the inclusion cyst and Fallopian tube hypotheses, other studies suggest low grade OCs, especially mucinous and endometrial, evolve from benign/ borderline neoplasia (Reviewed in [28]).
**Models of ovarian cancer.** Models of OC have been difficult to develop. Most animals do not develop spontaneous OC, with the exception of some strains of mice, rats, and hens. In these animals, the disease manifests with a low frequency and highly variable phenotype [29]. Other mouse models require manipulation, such as suppression of the large tumor suppressor 1 (Lats1) gene [29]. Normal HOSE can be immortalized with Large T antigen, and transformed with the human papilloma virus E6/E7 genes or the addition of E-cadherin to the Large T Antigen [29], however, characteristics of OC cell models vary with the oncogene used to transform the cells [30]. Transformed cells can then be injected subcutaneously or under the murine bursal membrane of the ovary to create animal models. These models have weaknesses, however, as humans do not have a bursal membrane, and as with most xenograft models, the host animal must be immunocompromised [30]. Resulting tumors in animal models frequently do not reflect the histologic subtype of the original tumor, resulting instead in undifferentiated tumors [30]). In contrast, intraperitoneal injection of cell lines derived from late stage human OC provides an excellent model for understanding OC invasion and metastasis [30], as OC metastases are most commonly found within the peritoneal cavity and represent the biggest barrier to improving OC survival.

Another major challenge to creating animal and cell models of OC is the lack of a clear molecular profile. Mutations, amplifications, and deletions have been found in multiple pathways, though each alteration is found in only a fraction of OC cases. Increased phosphatidylinositol 3-kinase (PI3K), Akt1, Akt2, estrogen receptor, and insulin-like growth factor 1(IGF-1)/IGF-1 Receptor (IGF-1R) activity have been
demonstrated in OC, as well as the loss of the tumor suppressor phosphatase and tensin homolog (PTEN) (Reviewed in [31]). Kirsten rat sarcoma viral oncogene homolog (kRas) is overexpressed in 30% of OCs, human epidermal growth factor receptor 2 (Her2) in 34%, and a subset of OCs exhibit p53 mutation or overexpression [6]. OC cells often exhibit acquired telomerase activity [32]. Despite these findings, however, no apparent universal culprit in OC initiation or progression has been elucidated.

**Risk factors for ovarian cancer development.** An effective strategy to increase the proportion of OC cases diagnosed earlier, and therefore better OC survival, is to analyze risk factors for OC development. Environmental factors such as use of talc on the genitals, cigarette smoking, radiation exposure, the use of certain medications, and diet have been associated with an increased OC risk [6]. Physiologic factors may also promote OC, for example obesity or history of another gynecologic disorder such as pelvic inflammatory disease, polycystic ovarian syndrome, or endometriosis [6]. Further, hormonal factors such as reproductive history and contraceptive use impact OC risk significantly. Early menarche and late menopause increase risk, while parity and breast feeding, as well as oral contraceptive use decrease OC risk [6]. The importance of hormone replacement therapy to OC risk is debated [33, 34]. The most informative risk factor, however, is current or past OC diagnosis of a first degree relative.

**BRCA1**

**Introduction.** Family history is the strongest risk factor for development of OC and a major risk factor for development of breast cancer [35]. Understanding how risk-associated mutations contribute to cancer initiation and progression will provide insight
into molecular mechanisms and aid in better risk assessment, prophylaxis, and treatment for carriers. The majority of hereditary OCs and a significant proportion of hereditary breast cancers are associated with mutation of the BRCA1 gene [35, 36].

**The BRCA1 gene product.** The 220 kDa, 1863 aa predominantly nuclear BRCA1 protein, which shuttles between the nuclear and cytoplasmic compartments, has multiple functions in the cell [37, 38]. BRCA1 plays an important role in the DNA damage response, as evidenced by the fact that BRCA1 null mice die early in embryonic development and exhibit chromosomal aberrations which are exacerbated by a p53 mutation [39], (Reviewed in [40] [41] [42]). BRCA1’s expression and phosphorylation are cyclic, and BRCA1 plays a role in the cell cycle as well, by regulating key cell cycle controllers, including cyclin dependent kinase inhibitor (p21), and by physically interacting with cell cycle regulators (Reviewed in [43]). BRCA1 can also recruit chromatin modifying proteins, such as histone acetyltransferases and histone deacetylases and directly interact with other transcription factors to alter their function (Reviewed in [43]). For example, BRCA1 binds and modulates phosphorylation of p53 to enhance its transactivation function [44] [45]. Lastly, BRCA1 is capable of ubiquitin ligase activity when heterodimerized with BRCA1-associated RING domain 1 (BARD1) [46]. The loss of these cellular functions of BRCA1 may contribute to cancer by promoting genomic instability and accumulation of cancer-causing mutations [40], a process further accelerated by p53 mutation, a common characteristic of BRCA1 mutant OCs [47]. BRCA1 mutation carriers have a 30% risk of developing OC during their lifetime [48] and a 50-80% risk of developing breast cancer before the age of 70 [40].
Types of BRCA1 mutations. All types of mutations have been reported in the 80 kb BRCA1 gene, including frameshift, nonsense, missense, in-frame insertions and deletions, splice altering mutations, mutations in the untranslated regions, as well as silent mutations. The majority of risk-associated mutations are frameshift or nonsense mutations that result in a premature stop codon and truncated protein product (NIH Breast Cancer Information Core). Risk-associated truncation mutations are found throughout the entire BRCA1 coding sequence (Figure 1.1) and result in mutant proteins that vary in length and structural impairment. For example, the nonsense mutation Y1853X, which lacks the last 11 amino acids, is only missing a small portion of the second (BRCA1 C-terminal) BRCT repeat, while the 39 amino acid 185delAG mutant lacks all of BRCA1’s known functional domains.

A smaller percentage of risk-associated BRCA1 mutations are point mutations classified as missense mutations. Like truncation mutations, missense mutations occur throughout the entire BRCA1 coding sequence (Figure 1.1) [49], though it is difficult to determine the clinical importance of these mutations because of their rarity and because they don’t often result in gross structural or functional loss. Therefore, many missense mutations remain “variants of unknown significance” [50]. The functional significance of the BRCA1 RING and BRCT domains as well as the substantial conservation of their sequences fuel speculation that many missense mutations in these areas are likely to be linked to cancer predisposition. Nonetheless, several missense mutations have already been linked to breast and/or OC predisposition including C61G, M1775K, and P1749R.

BRCA1 is thought to act as a classical tumor suppressor and loss of BRCA1’s cellular functions is thought to occur through bi-allelic inactivation. Carriers of mutations
**Figure 1.1.** BRCA1 mutations and their cellular and physiologic impact.

A. Domain structure of BRCA1 protein and location of risk-associated mutations. NES (Nuclear export signal), NLS (Nuclear localization signal).

B. BRCA1 mutations categorized by cellular processes in which each has been found to lack function or exhibit function different from wt. Nomenclature used for each mutation was that used in the original research article, or a structural description, if designation was not descriptive of mutation or mutant structure. (Linger and Kruk, 2010)

<table>
<thead>
<tr>
<th>DNA Damage Response</th>
<th>Apoptosis</th>
<th>Chemosensitivity</th>
<th>Proliferation</th>
<th>Transcription/ Gene Regulation</th>
<th>Tumorogenesis</th>
<th>Development</th>
</tr>
</thead>
<tbody>
<tr>
<td>5382insC</td>
<td>185delAG</td>
<td>185delAG</td>
<td>185STOP</td>
<td>185STOP</td>
<td>183STOP</td>
<td>trBRCA1 (N-term 3002aa)</td>
</tr>
<tr>
<td>M1775K</td>
<td>5382insC</td>
<td>5382insC</td>
<td>340STOP</td>
<td>340STOP</td>
<td>340STOP</td>
<td>Δ11 splice variant</td>
</tr>
<tr>
<td>P1749R</td>
<td>5677insA</td>
<td>N term 602aa</td>
<td>A43-1081*</td>
<td>A515-1092*</td>
<td>A750-1863*</td>
<td>W1777Stop*</td>
</tr>
<tr>
<td>C64G</td>
<td>AN</td>
<td>CT-BRCT1</td>
<td>AA1298-1863*</td>
<td>ANLS*</td>
<td>A513-1863*</td>
<td>ARING splice variant*</td>
</tr>
<tr>
<td>T826K</td>
<td>an303-1863*</td>
<td>N term 771 aa</td>
<td>A515-1092*</td>
<td>ANLSC-NLS*</td>
<td>A515-1091*</td>
<td>A542*</td>
</tr>
<tr>
<td>M1775R</td>
<td>AN</td>
<td>ΔK11m11</td>
<td>A500-1863*</td>
<td>ΔE101R1</td>
<td>A1808-5556*</td>
<td>BRCA1 tri/tr</td>
</tr>
<tr>
<td>AN an303-1863*</td>
<td></td>
<td>N terminal 1313aa*</td>
<td>ΔK11m11</td>
<td>N terminal 771aa</td>
<td>Ser1841Asn</td>
<td>a1-900*</td>
</tr>
</tbody>
</table>

* Denotes synthetic mutation
have one germline hit (the inherited mutated copy of BRCA1) and, in the tumor, a second somatic hit usually through loss of heterozygosity (LOH) [40]. The observed phenotype of enhanced breast and OC risk is generally thought to result from loss of some or all wild type (wt) functions of the BRCA1 gene product.

However, countless studies have revealed the complexities of signaling molecule and transcription factor interactions, as well as cellular adaptations in response to the unique selective pressures of tumor initiation and progression. Therefore, it is important to investigate all possible molecular mechanisms by which a mutation may contribute to the disease phenotype. Mutant proteins may antagonize wt proteins in a dominant negative manner resulting in loss of remaining wt function [51], or they may engage in unique molecular interactions and manifest novel functions independent of the loss of wt protein function [52]. Likewise, BRCA1 mutations may contribute to cancer risk through loss of wt BRCA1 function or through gain of function associated with mutant BRCA1 proteins.

**Loss of function mutations.** As mentioned previously, several lines of evidence suggest loss of wt BRCA1 function as a common mechanism for enhanced breast and OC risk (Table 1.1.). Similar to BRCA1 knockout mice and cell lines, elevated levels of aneuploidy and LOH indicative of an impaired DNA damage response have been noted in breast cancer tissue from mutation carriers compared to control breast cancers, as well as in the human BRCA1 truncated breast cancer cell line, HCC1937 (Reviewed in [40]). In structural protein studies, Tischkowitz et al. suggested that structural alterations in the BRCT phosphopeptide-binding pocket caused by the BRCA1 M1775K missense mutation contributed to enhanced breast and OC risk through diminished transactivation
Table 1.1. Studies supporting loss or gain of function mutation as mechanisms of enhanced BC and OC risk.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Result of Mutation</th>
<th>In vitro</th>
<th>In vivo</th>
<th>Model system</th>
<th>Endpoint</th>
<th>Summary</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Loss of Function</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Various</td>
<td>Missense P&gt;R</td>
<td>X</td>
<td>NA</td>
<td># genetic changes</td>
<td>Mutant BCs more chromosomal gain/loss events vs control BCs</td>
<td>Tirkkonen et al., 1997</td>
<td></td>
</tr>
<tr>
<td>P1749R C64G T826K M1775R</td>
<td>Missense C&gt;G Missense T&gt;K Missense M&gt;R</td>
<td>X</td>
<td>Breast cancer</td>
<td>DNA Damage</td>
<td>wt BRCA1 rescued γ radiation sensitivity of HCC1937 cells; Mutants did not</td>
<td>Scully et al., 1999</td>
<td></td>
</tr>
<tr>
<td>5382InsC</td>
<td>Truncated: 1828 aa</td>
<td>X</td>
<td>Breast cancer</td>
<td>DNA Damage, chemosensitivity</td>
<td>wt BRCA1 rescued hyper-recombination, chemosensitivity of MCF7 cells; Mutants did not</td>
<td>Cousins and Belmaaza, 2007</td>
<td></td>
</tr>
<tr>
<td>P1749R Q1756InsC Y1853stop</td>
<td>Missense P&gt;R</td>
<td>X</td>
<td>COS, colon cancer</td>
<td>Gene regulation</td>
<td>wt BRCA1 increased p21 expression in COS, cancer cells; Mutants did not</td>
<td>Somasundaram et al., 1997</td>
<td></td>
</tr>
<tr>
<td>1835STOP 340STOP</td>
<td>Truncated: 1834 aa Truncated: 339 aa</td>
<td>X</td>
<td>Breast cancer</td>
<td>Cell growth, tumor growth</td>
<td>wt BRCA1 inhibited growth, tumor growth in nude mice; Mutants did not</td>
<td>Holt et al., 1996</td>
<td></td>
</tr>
<tr>
<td>Gain of Function</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5677InsA</td>
<td>Truncated: 1852 aa</td>
<td>X</td>
<td>Prostate cancer</td>
<td>Proliferation</td>
<td>Mutant inhibited proliferation more efficiently than wt BRCA1</td>
<td>Fan et al., 1998</td>
<td></td>
</tr>
<tr>
<td>N terminal 602 amino acids</td>
<td>Synthetic mutant: 602 aa</td>
<td>X</td>
<td>Mouse ovarian epithelium</td>
<td>Proliferation, chemosensitivity, tumorigenesis</td>
<td>Mutant BRCA1 enhanced proliferation, chemosensitivity, tumorigenesis; wt BRCA1 suppressed</td>
<td>Sylvain et al., 2002</td>
<td></td>
</tr>
<tr>
<td>5677InsA N terminal 302 aa N terminal 771 aa</td>
<td>Truncated: 1852 aa Synthetic mutant: 302 aa Synthetic mutant: 771 aa</td>
<td>X</td>
<td>Prostate cancer</td>
<td>Proliferation, cho-mo-sensitivity</td>
<td>5677InsA and wt BRCA1 impaired proliferation, enhanced chemosensitivity; Synthetic truncations decreased sensitivity</td>
<td>Fan et al., 2001</td>
<td></td>
</tr>
<tr>
<td>185delAG</td>
<td>Truncated: 39 aa</td>
<td>X</td>
<td>Ovarian epithelium</td>
<td>Apoptosis</td>
<td>185delAG decreased cIAP1, XIAP, P-Akt, and enhanced cleaved caspase 3, apoptosis after drug treatment</td>
<td>O’Donnell et al., 2008</td>
<td></td>
</tr>
<tr>
<td>5382InsC 5677InsA</td>
<td>Truncated: 1828 aa Truncated: 1852 aa</td>
<td>X</td>
<td>Breast, Ovarian Cancer</td>
<td>Apoptosis</td>
<td>Coexpression of mutants with wt BRCA1 inhibited wt BRCA1’s ability to enhance apoptosis</td>
<td>Thangaraju et al., 2000</td>
<td></td>
</tr>
<tr>
<td>5083del19</td>
<td>Truncated: 1669 aa</td>
<td>X</td>
<td>X</td>
<td>HeLa</td>
<td>Gene regulation</td>
<td>Mutant increased periostin mRNA, protein, and mutation carrier serum, BC tissue</td>
<td>Quaresima et al., 2008</td>
</tr>
</tbody>
</table>

(Linger and Kruk, 2010)
and binding to other DNA damage response proteins [53]. Likewise, Williams et al. found that decreased stability of BRCA1 missense and truncation mutants resulting from aberrant protein folding contributed to loss of BRCA1 function and enhanced cancer risk [54].

Expression of mutant BRCA1 constructs in the absence of wt BRCA1 frequently fails to restore wt BRCA1 function. Scully et al. utilized the γ radiation sensitive HCC1937 breast cancer cell line, which lacks wt BRCA1 and carries two 5382InsC BRCA1 alleles that code for a frameshift and premature stop signal at codon 1829, and were able to decrease γ radiation sensitivity with restoration of wt BRCA1. However, transfection of several BRCA1 mutants into these cells failed to alter radiation sensitivity [55]. In agreement, addition of wt BRCA1 expression into breast cancer cell lines that exhibit low wt BRCA1 expression due to the presence of a single wt BRCA1 allele inhibited growth. However, expression of the risk-associated truncation mutants 1835STOP and 340STOP as well as the synthetic internal deletion mutants Δ343-1081 and Δ515-1092, failed to alter cell growth, tumor formation and tumor progression in nude mice [56]. Lastly, introduction of wt BRCA1 into HCC1937 breast cancer cells and IGROV 1 OC cells inhibited tumor initiation and growth, while a synthetic BRCA1 mutant lacking the last 542 amino acids did not [57]. Interestingly, Cousineau and Belmaaza hypothesize that reduced gene dosage of wt BRCA1 in mutation carriers is solely responsible for altered DNA damage repair, subsequent mutation accumulation, and increased cancer risk. Using MCF7 breast cancer cells that harbor a single copy of wt BRCA1 and exhibit enhanced spontaneous recombination or “hyper-recombination,” Cousineau and Belmaaza showed that transfection of MCF7 cells with wt BRCA1
diminished hyper-recombination and chemosensitivity while addition of the 5382InsC BRCA1 mutation affected neither endpoint [58]. These studies further support a role for loss of wt BRCA1 function as a contributing factor to enhanced breast and OC risk.

It is important to note that many of the aforementioned studies attempted to delineate BRCA1 mutant function in model systems lacking normal levels of wt BRCA1, which makes it difficult to discriminate between the contribution of BRCA1 mutants and loss of wt BRCA1 for disease risk. However, several studies utilizing a wt BRCA1 background clearly support the loss of BRCA1 wt function for cancer risk. For example, though overexpression of wt BRCA1 in several wt BRCA1 cancer cell lines and COS cells up-regulated p21 expression, several synthetic deletion and truncation mutants and risk-associated BRCA1 mutants, including P1749R, Q1756InsC (aka 5382InsC), and Y1853STOP (aka 5677InsA), a frameshift mutation resulting in a premature stop codon that lacks the last 11 amino acids [59], failed to alter p21 expression [60].

**Gain of function mutations.** While mutations resulting in a premature stop codon are typically susceptible to nonsense-mediated messenger RNA (mRNA) decay, mounting evidence suggests mutant mRNA and proteins are not uniformly degraded. Perrin-Vidoz et al. found that several BRCA1 mutations were unaffected by mRNA decay, including 185delAG and 5382InsC [61], two of the most common risk-associated BRCA1 mutations [62]. Truncation mutant mRNAs may avoid decay by translation reinitiation at a methionine codon downstream of the premature stop codon [63], and consequently, may contribute aberrant gene products coding for truncation proteins exhibiting varying degrees of protein stability that may impart novel cellular functions [64]. It is important to consider that detection of some mutant BRCA1 proteins in clinical
samples has proven unsuccessful due to technical challenges such as cross-reactivity of antibodies with wt BRCA1, however, validation studies of mutant proteins in tissue samples are ongoing and will provide a framework within which to view experimental studies of mutant function.

BRCA1 mutant proteins may participate in novel protein-protein interactions as a result of aberrant cellular localization. Rodriguez et al. found that exogenous missense and truncation mutants lacking a small portion of the BRCA1 C terminus, including 5382InsC, exhibited aberrant cytoplasmic localization in breast cancer cells, while larger truncations resulted in enhanced nuclear localization of mutants [65]. Aberrant localization may result from mutation or loss of the nuclear localization or export signals, impaired recognition of these signals as a result of improper protein folding, or altered interaction with binding partners that impact BRCA1 localization, such as BARD1 [65].

Mutant BRCA1 proteins may convey unique phenotypes by inhibiting normal function of wt BRCA1 in a dominant negative manner by binding BRCA1 and inhibiting its interaction with other proteins, or by sequestering BRCA1 binding partners. Likewise, mutant proteins may also convey unique functions by interacting with novel proteins and/or regulating alternative genes. Indeed, a significant proportion of BRCA1-associated breast cancer tissue samples [66], as well as primary cells from mutation carrier-derived OC cell xenograft tumors [67], exhibit loss of the wt BRCA1 allele concomitant with increased mutant allele copy number. Consequently, mutant BRCA1 proteins have been shown to impact a range of cellular functions including development, proliferation, chemosensitivity, apoptosis, and gene regulation (Table 1.1.).
Role of gain of function mutations for development, cellular proliferation, chemosensitivity, apoptosis and gene regulation. Essentially all BRCA1 knockouts are embryonic lethal in mice (Reviewed in [68]), however, mice homozygous for a specific synthetic mutation truncating the BRCA1 protein by half are viable, though highly susceptible to multiple tumor types, including lymphomas, sarcomas, and carcinomas/adenocarcinomas of the colon, endometrium, lung, liver, and mammary gland [69]. Interestingly, introduction of a synthetic BRCA1 truncation mutant encoding the first 300 BRCA1 amino acids inhibits mammary gland differentiation and structural formation during murine development, despite the presence of wt BRCA1 [70]. Likewise, when injected into the cleared murine mammary fat pad, primary human breast epithelial cells transfected with the BRCA1 Δ11 splice variant or murine BRCA1-W1777Stop, (which mimics the human 1835STOP mutation), undergo limited differentiation and branching and develop extensive hyperplasia [71].

The 5677InsA insertion mutation, resulting in a frameshift and premature stop signal at codon 1853, inhibits proliferation of DU145 human prostate cancer cells expressing a low level of wt BRCA1 more efficiently than exogenous wt BRCA1 [72], while a synthetic N terminal mutant was found to inhibit physical interaction of wt BRCA1 and cyclin D1 [73]. In contrast, an exogenous C terminal fragment of BRCA1 can enhance normal breast epithelial cell growth, possibly by acting in a dominant negative manner to inhibit wt BRCA1’s growth suppressive function [74]. Similarly, while overexpression of wt BRCA1 in the ID8 mouse ovarian epithelial cell line diminished proliferation, chemosensitivity, and tumorigenicity of intraperitoneally injected cells, expression of a synthetic truncation mutant encoding the first 602 amino
acids of BRCA1 yielded enhanced proliferation and chemosensitivity. Further, when injected intraperitoneally, cells expressing the mutant were significantly more tumorigenic [75]. It should be noted, however, that BRCA1 mutants have also been shown to exhibit some residual wt growth function as a result of remaining intact domains. For example, mouse embryonic fibroblasts homozygous for Δ11 BRCA1 exhibited a failed G2-M checkpoint [76], while breast cancer cells expressing only the 5382InsC mutant maintained an intact G2-M checkpoint [55].

In DU145 prostate cancer cells expressing low levels of wt BRCA1, Fan et al. reported that overexpression of wt BRCA1 or 5677InsA increased topoisomerase inhibitor cytotoxicity, which could be reversed by transfection of synthetic mutants ΔEcoRI (amino acids (aa) 1–302) and ΔKpnI (aa 1–771), yielding chemoresistant cells [73]. Likewise, in the HCC1937 breast cancer cell model system lacking endogenous wt BRCA1, addition of exogenous wt BRCA1 enhanced chemoresistance, which was reversed by co-transfection of ΔEcoRI and ΔKpnI [73]. This suggests that mutants can, at least in part, overturn wt BRCA1 function, thereby supporting a role for gain of function BRCA1 mutations.

The 185delAG (BRAT) mutation, which imparts upon carriers a 66% lifetime risk of developing OC [77], arises from the deletion of two nucleotides (AG) in the second exon of the BRCA1 gene. This deletion results in a reading frame shift that produces a premature stop signal at codon 39 and a truncated protein product. Using SV-40 transfected HOSE cells from women with the BRAT mutation, we found that mutant cells exhibited enhanced apoptosis and caspase 3 activation in response to staurosporine [78], possibly related to diminished levels of phospho-Akt/protein kinase B, X-linked
inhibitor of apoptosis protein (XIAP), and cellular inhibitor of apoptosis protein 1 (cIAP1) [79]. To rule out the possible contribution of wt BRCA1 haploinsufficiency to altered apoptosis in 185delAG cells, BRAT was expressed in wt BRCA1 HOSE cells. In agreement with our earlier studies, BRAT enhanced caspase3-mediated apoptosis and diminished levels of phospho-Akt, cellular inhibitor of apoptosis 1 (cIAP1), and x-linked inhibitor of apoptosis 1 (XIAP) [80]. In more recent studies, we found that BRAT upregulated expression of maspin [81], a tumor suppressor important in apoptosis, invasion, and metastasis that is uniquely overexpressed in several tumor types, including OC [82]. Maspin expression has been correlated with cisplatin sensitivity in OC cell lines and longer progression-free and overall survival times in OC patients [83], and may be involved in BRAT-mediated enhanced chemosensitivity [81]. Lastly, Thangaraju et al. found that co-expression of 5382InsC and 5677InsA with wt BRCA1 inhibited the wt protein’s ability to enhance apoptosis in breast and OC cells [84].

Several studies support a role for BRCA1 mutants in gene regulation. For example, wt BRCA1 and 5677InsA inhibited exogenous estrogen receptor alpha transactivation, but co-transfection of ΔBamHI, ΔKpnI, and ΔEcoRI reversed this phenomenon [73]. Similarly, the synthetic BRCA1 mutant (△500-1863), which encodes a protein less than a third the length of wt, inhibited wt BRCA1-mediated activation of a p53 reporter [44]. Likewise, using the mouse mammary gland-specific expression of wt BRCA1, a risk-associated mutation that truncates the protein at amino acid 340, or a BRCA1 splice variant that omits the N-terminal 72 amino acids, Hoshino et al. showed that the splice variant mediated hyperproliferation and enhanced lobule formation in the mammary gland. In addition, tumorigenesis and death were accelerated in mice
expressing the splice variant [85]. In separate studies, Quaresima and colleagues performed microarray analysis on HeLa cells stably expressing vector, wt BRCA1, or the founder mutation 5083del19, which encodes a BRCA1 protein missing the last 193 amino acids, and, consequently both BRCT domains, and found differential regulation of multiple genes, including up-regulation of periostin. Further, periostin levels were also increased in serum and breast cancer tissue from a small number of patients carrying this mutation [86]. In other studies, expression of a synthetic truncation mutant maintaining the first third of the BRCA1 protein enhanced p53 expression in 1D8 mouse epithelial OC cells and down-regulated constituents of the c-Jun N-terminal kinase/stress activated protein kinase (JNK/SAPK) and mitogen activated protein kinase/ extracellular signal regulated kinase (MAPK/ERK) pathways [87]. Finally, the missense mutation Ser1841Asn, which is associated with enhanced breast cancer risk, up-regulates tumor protein D52 (TPD52) and the folate receptor alpha (FOL1) in HeLa cells [88]. This regulation is clinically relevant since expression of these genes correlates with tumor progression in breast [89] [90] and OCs [91] [92].

Taken together, these studies support a gain of function role for some mutations. The presence or absence of a mutant function as well as its impact on the cell is likely very specific to each mutation and factors impacting mutant function, including mutant protein size, loss/ maintenance of various domains, or structural changes resulting in novel domains. These studies must also be viewed in a cautionary manner. Gain and loss of function experiments provide valuable insight into the mechanism of BRCA1 mutant functions, however, until the presence of stable mutant proteins is validated clinically, it
is necessary to remain mindful of the limitations as well as the promise of these types of experimental studies.

**Clinical impact of gain of function mutations.** Studies investigating the effect of BRCA1 mutant proteins in the context of wt BRCA1 are clinically important. They represent the genotypic and phenotypic state of disease-free mutation carriers before loss of both wt BRCA1 alleles. Novel functions mediated by mutant proteins have been shown in various model systems to significantly impact proliferation and apoptosis, and therefore, have the potential to influence cancer initiation, progression, and ultimately prognosis for patients carrying mutations. While some mutants may retain specific wt BRCA1 functions, others may enhance the risk of cancer development by antagonizing BRCA1’s tumor suppressive functions. Further investigation of mutant protein function is warranted, as a better understanding of the function of specific mutations could greatly improve risk assessment and prognostic value for mutation carriers.

A better understanding of BRCA1 mutant functions may also help identify novel drug targets for treatment and prophylaxis of mutation carriers. Novel interacting proteins and signaling pathways as well as downstream target genes may reveal as-yet unidentified players in BRCA1 mutation-associated breast and OC. Data from our lab suggests that genes important for cancer initiation and progression, such as maspin, are differentially regulated in normal human ovarian epithelial cells expressing the BRAT mutation [81]. Further, compared to sporadic breast cancer tissue, BRCA1 mutation-associated breast cancer samples reveal more chromosomal aberrations in specific regions, potentially containing additional tumor suppressors important in BRCA1-dependent tumor initiation and progression [93]. An understanding of specific interacting
proteins, signaling pathways, and target genes involved in the mechanism of enhanced breast and OC risk conveyed by each mutation provides the opportunity for mutation-specific personalized therapy for mutation carriers. Similar mutations may also share common functions and respond to similar therapeutic strategies. Further, targeting functions of BRCA1 mutants that likely contribute to pre-malignancy, cancer initiation, and early stages of tumor growth holds great promise for effective prophylactic measures that are less invasive than oophorectomy and mastectomy.

It is interesting to speculate that cells heterogeneous for risk-associated mutations, though non-tumorigenic in their current state, may represent an initial step toward cellular transformation, though additional changes may be necessary for these cells to become malignant. Likewise, early changes that may promote malignant transformation, including enhanced telomeric instability, have been observed in cell lines generated from normal ovarian surface epithelial cells of women with a strong family history of OC [94] (Reviewed in [95]). As mentioned previously, several studies have found more frequent occurrence of deep invaginations in the ovary surface, dysplasia, hyperplasia, and/or surface papillae in high risk prophylactically removed ovaries versus normal ovaries [16] [17] [18], suggesting that early “pre-malignant” changes may already exist in those carriers. The possibility of independent mutant BRCA1 functions does not exclude the contribution of other oncogenes, tumor suppressors, or invasion/metastasis-promoting proteins. Conversely, these early changes are likely to facilitate further cellular changes that manifest in the aggressive phenotype seen clinically in hereditary breast and OC.

Though the specificity of BRCA1 mutations for increasing the risk of breast and OC is well established, few studies have investigated differences in risk and etiology
between BRCA1 mutation-associated breast and OC. This question warrants investigation, as the lifetime risk for development of breast cancer is higher than that for OC [14] [96], and BRCA1 mutation carriers don’t always develop both breast and OC. Further, families with predisposition to both breast and OC exhibit variation in the ratio of breast to OC occurrence [97], and this ratio is dependent in part on the location of the mutation within the BRCA1 gene (Reviewed in [98]). Disparate risk levels of breast and OC suggest mutant proteins may mediate different functions in different tissues, which in turn may exhibit tissue-specific degrees of importance for the specific functions lost or gained as a result of each mutation.

In conclusion, it is clear from a wide range of model systems and endpoints that BRCA1 mutations are capable of significant physiologic impacts. Further, molecular and phenotypic changes are evident in mutation carriers. These changes may result from loss of wt BRCA1 function, gain of function mutations, or both. Consequently, further experimental and clinical studies of mutant BRCA1 proteins are warranted, and will provide a better understanding of mutation-associated breast and OC and improve the strength of prognosis and efficacy of prophylaxis and treatment for mutation carriers.

**MMP1**

**Introduction.** The aggressive clinical course of OC illustrates the importance of invasion and metastasis in OC progression. One of the normal physiologic processes that cancer cells frequently exploit to attain these capabilities is cleavage of extracellular matrix (ECM) components and other substrates by matrixmetalloproteases (MMPS). This adaptation that can ultimately promote tumor growth, invasion, and metastasis through
multiple mechanisms (Reviewed in [99]. Preliminary microarray data from our lab suggest that the gene encoding MMP1 may be differentially regulated in HOSE cells expressing BRAT.

**Structure and function.** MMPs are a family of proteases that utilize Zinc at their active site to cleave a wide variety of substrates. Specifically, MMP1 cleaves fibrillar collagens, but has also been shown in vitro to cleave other substrates, including ECM constituents, pro-growth factors, growth factor receptors, and cell-cell adhesion mediators [100]. The N-terminal 18-30 amino acids of MMPs comprise a signal peptide (Figure 1.2.), which directs translation of the protein to the endoplasmic reticulum, and is cleaved off before secretion [101]. The MMP “pro domain” contains a critical cysteine residue (amino acid 73 of MMP1), that occupies the active site zinc with a sulfhydryl group, thereby rendering the enzyme inactive [102]. The catalytic domain is comprised of a five stranded beta sheet and two alpha helices [102], 160-170 amino acids, and contains the sequence HELGHXXGXXH [101]. The three histidines within this sequence bind the catalytic zinc molecule and are crucial for enzymatic function [101]. MMPs bind one structural zinc and a calcium as well. The hinge, or linker, domain follows. This region is variable in length and sequence in MMPs and is thought to be involved in disrupting the triple helix structure of collagen I to increase accessibility of the cleavage site. Lastly, the structure of the C-terminal domain varies, and contains a transmembrane spanning region in some MMPs. MMP1 is classified structurally by its C terminus as a simple hemopexin domain family member. This domain, comprised of four repeat regions that form a four-bladed beta propeller [102], is similar in sequence to vitronectin, and may mediate
# Matrixmetalloprotease I

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Potential importance in OC</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen I</td>
<td>Present in ovarian &amp; omental extracellular matrix (ECM)</td>
<td>Kenny et al., 2008</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ricciardelli and Rodgers, 2006</td>
</tr>
<tr>
<td>Collagen III</td>
<td>Present in ovarian ECM</td>
<td>Ricciardelli and Rodgers, 2006</td>
</tr>
<tr>
<td>Entactin/niidogen</td>
<td>Present in ovarian ECM</td>
<td>Ricciardelli and Rodgers, 2006</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>Present in ovarian &amp; omental ECM</td>
<td>Kenny et al., 2008</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ricciardelli and Rodgers, 2006</td>
</tr>
<tr>
<td>Laminin</td>
<td>Present in ovarian &amp; omental ECM</td>
<td>Kenny et al., 2008</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ricciardelli and Rodgers, 2006</td>
</tr>
<tr>
<td>Tenascin</td>
<td>Present in ovarian ECM</td>
<td>Ricciardelli and Rodgers, 2006</td>
</tr>
<tr>
<td>Pro-MMP2</td>
<td>MMP2: Cleaves collagen IV (major component of ovarian basal lamina) and other ECM components, Promotes OC metastasis</td>
<td>Kenny et al., 2008</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ricciardelli and Rodgers, 2006</td>
</tr>
<tr>
<td>Pro-MMP9</td>
<td>MMP9: Cleaves multiple ECM components</td>
<td>Kenny et al., 2008</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ricciardelli and Rodgers, 2006</td>
</tr>
<tr>
<td>Pro-TNFα</td>
<td>TNFα: Produced by OSEs and OC cells; Increases MMP1 expression in human dermal fibroblasts; Upregulates MMP2 expression; Enhances proliferation, protease secretion, and decreases contact inhibition of OC cells in vitro</td>
<td>Auersperg et al., 2001</td>
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<td></td>
<td></td>
<td>Dayer et al., 1985</td>
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<td></td>
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<td>Murdoch and McDonnel, 2002</td>
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</tbody>
</table>

**Figure 1.2.** MMP1 domain structure and potential substrates of importance in OC.
binding to ECM components, and/or help lock collagen into the active site for more
efficient cleavage [101].

MMPs are synthesized and secreted as zymogens. In the inactive form, the
cysteine residue within the pro domain inhibits activation by binding the catalytic zinc
molecule, preventing its interaction with a water molecule that is crucial for catalysis
[101]. Pro MMPs can be activated by mercurial compounds (such as 4-
aminophenylmercury acetate (APMA)), other thiol reactive reagents, reactive oxygen
species, heat treatment, and other compounds by occupation of the cysteine and
disruption of the cysteine-zinc interaction. Biologically, MMPs are activated by other
MMPs and proteases, such as trypsin, through cleavage and partial removal of the pro
domain, thereby freeing the active site zinc. MMP1 subsequently autocleaves to remove
an additional portion of the pro domain, however, this 42 kilodalton (kDa) MMP1 is only
about 20% active. MMP1 is fully activated by cleavage at residue 80 into a 41 kDa
product. This may be achieved by MMP2, 3, 7, 10, or 11. In the absence of one of these
proteases, MMP1 may further autocleave at residue 81 or 82, resulting in a 30-40% active
enzyme [101].

MMPs cleave a variety of physiologically important substrates, but do not cleave
at a strict consensus sequence. In fact, there are few specific characteristics shared by
substrates. MMP1 is one of a small group of proteases capable of cleaving native, or
fibrillar, collagens, which are the most abundant collagens in interstitial connective tissue
[103]. The ovarian stroma is composed mostly of collagens I and III and fibronectin,
while fibulin 1, perlecan and other heparin sulfate proteoglycans (syndecans and
glypicans), lecticans, decorin, and hyaluronan chains are also present (Reviewed in
At the surface of the ovary, beneath the HOSE lies the basal lamina, which is comprised primarily of networks of collagen type IV and laminin, as well as nidogen/entactin and perlecan [104]. A dense network of collagen fibrils called the tunica albuginea underlies the basal lamina. MMP1 substrates with potential importance in OC pathology are shown in Figure 1.2. As frequent metastasis is a highly important feature of OC, and the omentum is the most common site of OC metastases [105], the occurrence of MMP1 substrates in the omental ECM and stroma are also listed. Though these substrates have been identified in vitro, their importance in vivo is still being investigated.

**Regulation.** MMPs are regulated by proteolytic activation, as mentioned previously, as well as through pericellular localization. MMPs can be regulated by localized availability of activators, such as the membrane-associated urokinase plasminogen activator (uPA), while other MMPs are membrane bound.

MMP family members are also regulated by expression. Transcriptional regulation of MMP-1 occurs in part through activator protein 1 (AP1) [106] and v-ets erythroblastosis virus E26 oncogene homolog 1 (Ets) [107] sites in the promoter. The most proximal AP1 site is located at (-73) and is necessary for basal transcription, though other AP1 and Ets sites upstream also contribute to basal transcription. IL-1 and TNFalpha-mediated up-regulation occurs through c-Jun and an AP1 site as well, though there may also be promoter regions through which IL-1 negatively impacts expression. Expression of MMP1 is also modulated by other cytokines and growth factors. For example, platelet-derived growth factor (PDGF) and EFG up-regulate MMP1, while transforming growth factor β (TGFβ), retinoids and glucocorticoids inhibit expression [108]. Epidermal growth factor (EGF) and interleukin 1β (IL-1β) are known to enhance
MMP1 mRNA stability [109, 110]. These impacts are cell type specific, however, and factors may exert alternative context-dependent effects.

MMPs are also up-regulated by Extracellular Matrix Metalloprotease Inducer, or EMMPRIN. EMMPRIN, a member of the immunoglobulin superfamily, is heavily glycosylated and localizes to the cell surface. Staining for EMMPRIN is frequently observed at the leading invasive edge of tumors [111]. EMMPRIN was originally isolated from the membranes of cancer cells, however, it is secreted by and affects both cancer and normal stromal cells. Transfection into breast cancer cells increases tumorigenicity in nude mice and expression of MMP2 and 9 in tumors [112]. Expression is elevated in malignant ovarian tumors compared to normal tissue [113], and correlates with poor survival in serous OC [114]. The mechanism by which EMMPRIN induces MMP expression has not been completely elucidated, though clustering of EMMPRIN with itself and caveolin 1 has been implicated, as well as signaling through p38 MAPK and JNK [115] [116].

MMPs are regulated extracellularly by the binding of several protein types. The tissue inhibitors of metalloproteinases (TIMPS) are “wedge-shaped” molecules that bind MMPs in a 1:1 ratio and displace the water molecule usually associated with the catalytic zinc [101]. Specifically, MMP1 is inhibited by all four TIMP proteins (1-4) [101]. The importance of TIMPs in some types of cancer has been illustrated. Melanoma cells overexpressing TIMP3 exhibit decreased invasive ability and undergo apoptosis [117]. TIMP secretion from stromal cells also influences MMP activity greatly, as brain metastases of fibrosarcoma cells were significantly diminished in mice with brain-specific overexpression of TIMP1 [118]. MMPs are also inhibited by the large serum
protein α2 macroglobulin (α2M). α2M binds MMPs after they cleave a “bait” peptide. α2M then undergoes a conformational change that prohibits access of substrates to the MMP [108], and the complex is subsequently endocytosed [101].

**MMPs and cancer.** MMPs are involved in normal physiologic processes, including wound healing, mammary gland and uterine involution, and cervical dilation [119]. In the ovary, MMPs are up-regulated in the pre-ovulatory follicle [120] and are secreted by ovarian surface epithelial cells from lysosomes [121]. Both sources of proteases may aid in release of the oocyte during ovulation.

Conversely, MMPs have been implicated in cancer. Mutations and gene amplifications are not generally reported, but MMP expression increases in multiple cancer types, including ovarian [99]. Microarray data suggest MMP12 may be a useful serum marker for breast cancer [122], while MMPs 2 and 19 may be useful biomarkers for OC [123]. Specifically, MMP1 expression has been associated with poor prognosis in breast, colorectal, and esophageal cancer [124-126].

MMPs are crucial for invasion of tumor cells into and through the basement membrane, thereby promoting metastasis [127]. Cleavage of ECM constituents may reveal binding sites in the ECM that modulate migration and adhesion. MMPs also cleave and release pro-growth factors that may promote tumor cell growth (Reviewed in [99]). Kenny et al. found that adhesion of SkOV3 OC cells to mouse peritoneum and a 3-D peritoneal model decreased after MMP2 inhibition or knock down, and that MMP2 expression was enhanced in adherent cells [105].

MMP family members also participate in multiple novel mechanisms of cancer progression, including angiogenesis, apoptosis, and the immune response to cancer
(Reviewed in [99]). The importance of MMPs in angiogenesis is especially relevant to OC. ECM remodeling allows migration of endothelial cells through the ECM. MMPs also cleave and release pro- and anti-angiogenic factors from the ECM (Reviewed in [99]). Interestingly, recent studies have also demonstrated the ability of aggressive OC cells to form vascular-like structures in 3D culture, and integrate into the tumor vasculature in a phenomenon called “vascular mimicry.” Several MMPs, including MMP1 have been detected within the vascular structures [128], and inhibiting MMPs diminishes the formation of vascular structures by the OC cells [129].

In addition to interactions of tumor cells with the ECM, stromal-tumor cell interactions are known to be important in multiple facets of tumor progression, including paracrine growth signaling and angiogenesis. For example, normal sheep ovarian stromal cells were found to inhibit the growth of OC cell lines in vitro and in vivo [130]. Conversely, tumors formed from subcutaneously injected OC cells exhibited regions of host stromal tissue integrated into the tumor that could promote further tumor growth [131]. Mouse ovarian stromal cells stimulated anchorage-independent growth of HOSE cells [31]. Co-culture also enhanced estrogen-stimulated growth of rabbit OSE cells [132]. It is possible that TIMPS or MMP regulators may be secreted by ovarian stromal cells in vivo. This phenomenon has been examined in detail in a melanoma cell model that highly expresses MMP1. Melanoma cells exhibited invasion through a type I collagen matrix only when co-cultured with fibroblasts or treated with fibroblast conditioned media. MMP3 and an unidentified serine protease secreted by the fibroblasts cleaved and activated MMP-1 secreted by the melanoma cells. The activated MMP1 subsequently facilitated invasion [133].
The specific pathological role for matrix metalloprotease 1 in OC has not yet been made clear. Knockout mice of several other MMPs have been studied, and reveal a general theme of decreased tumorigenesis and angiogenesis [134]. Unfortunately, however, there is not a sufficiently similar homologue to MMP1 in the mouse genome, so an MMP1 knockout mouse has not yet been created. However, using intraperitoneal injection of human OC cells into mice, Agarwal et al. implicate MMP1 in the activation of protease activated receptor 1 (PAR-1) and reveal the importance of this pathway to ascites formation, metastasis, and angiogenesis [135].

In a 1998 study utilizing a leukocyte gene library, a single nucleotide polymorphism (SNP) was found in the MMP1 promoter. The insertion of one “G” nucleotide was found to increase the efficacy of an Ets (erythroblastosis virus E26 oncogene homolog) transcription factor binding site and increase MMP1 expression [136]. A later study found that the percentage of OC patients with the SNP was disproportionately high compared to normal women, and that tumors with the 2G allele expressed greater than seven times the MMP1 expression levels of tumors without a 2G allele, suggesting that MMP1 is indeed important in OC pathology [103]. Further, presence of the 2G polymorphism was associated with decreased disease-free and overall survival in OC patients [137].

**Rationale**

We have previously found that BRAT plays an important role in the enhanced apoptotic response of immortalized HOSE cells to drug treatment. Immortalized HOSE cell lines derived from BRAT mutation carriers exhibit enhanced caspase 3 activation and apoptosis as a result of diminished levels of apoptotic inhibitors XIAP and cIAP1 and
reduced Akt activation [78, 79]. Enhanced chemosensitivity is mediated by the BRAT mutation, independently of the loss of wt BRCA1, as transfection of BRAT into wt HOSE and OC cells recapitulates the effects on XIAP, caspase 3, and apoptosis [79, 80]. These findings are in agreement with studies illustrating better initial chemotherapeutic response in BRCA1 mutation carrier OC patients compared to control patients [138, 139].

More recently, we have identified the tumor suppressor maspin as a downstream target of BRAT. Maspin mRNA and protein levels are increased in wt 118 HOSE cells transiently and stably expressing BRAT. Maspin up-regulation is transcriptional, and occurs in part through c-Jun, as maspin promoter activity is significantly decreased by c-Jun knockdown as well as truncation of the maspin promoter and elimination of several transcription factor binding sites, including an AP1 site [81]. c-Jun is critical for enhanced BRAT cell chemosensitivity as well. c-Jun knockdown decreases caspase 3 cleavage [81]. Preliminary data also suggest maspin plays a role in BRAT cell chemosensitivity, as maspin knockdown results in diminished caspase 3 cleavage as well (Figure 1.3., [81]). These findings are clinically relevant, as cytoplasmic maspin correlates with cisplatin sensitivity in OC cell lines, and treatment response and overall survival in OC patients [83].

It is clear from these studies that the BRAT mutation mediates unique molecular and cellular changes in HOSE and OC cells independent of the loss of wt BRCA1 function, and that these changes have great potential for physiologic impact in carriers of the BRAT mutation. Consequently, it is worthwhile to pursue identification of additional BRAT targets and cellular processes important in OC initiation and progression. The
Figure 1.3. Maspin, in part, mediates the enhanced apoptotic response of BRAT cells to staurosporine (STS) treatment.
Stable BRAt cells were transfected with Si Con or Si Maspin, and 48 hours later, were treated with 1 µM STS. Lysates were collected six hours after treatment. Samples were analyzed for cleaved caspase-3, pro-caspase-3, maspin, and actin protein levels via western blot. (O’Donnell et al., 2010)
185delAG mutation is associated with increased risk of breast cancer risk as well as OC, however mutation-associated breast and OC occur with different penetrance in families and individuals. Therefore, it is also important to determine whether the cellular functions of BRAT are tissue specific and could contribute to epidemiologic and etiologic differences between 185delAG-associated breast and OC.

**Central Hypothesis**

I hypothesize that expression of the 185delAG BRCA1 mutant protein product, BRAT, alters the regulation and/or activity of several potentially important players in BRAT-associated OC pathology, including matrix metalloprotease 1. I hypothesize that BRAT-mediated MMP1 up-regulation involves Akt and the transcription factor c-Jun, and occurs in part through specific AP1 sites in the MMP1 promoter. I also hypothesize that BRAT mediates differential effects on apoptosis, gene regulation, and migration in normal HOSE and ovarian cancer cells compared to normal breast epithelial and breast cancer cells. BRAT-mediated molecular and cellular changes in ovarian surface epithelial cells may serve, then, as an intermediate state in the transformation of these cells from normal to malignant.

**Specific Aims**

To address these hypotheses, I propose execution of three specific aims.

1. Confirm MMP1 as a downstream target of BRAT in immortalized human ovarian surface epithelial cells.

2. Determine the signaling pathways and transcription factors involved in BRAT-mediated MMP1 up-regulation.

3. Demonstrate the specificity of BRAT function in ovarian compared to breast cancer by comparison of BRAT’s impact on apoptosis, gene regulation, and migration in normal human breast epithelial cells and breast cancer cells.
References


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Chapter 2:
The 185delAG BRCA1 Mutation Enhances MMP1 Expression in Human Ovarian Surface Epithelial Cells

Introduction

Ovarian cancer is the 9th most common cancer in women, but ranks 5th in cancer related deaths [1]. The deadliness of this disease can be attributed in part to the fact that early stages of OC are not well understood and are virtually asymptomatic. The majority of OC diagnoses are made in Stage 3 and 4, when the primary tumor has metastasized and patient survival falls below 30% [1]. In stark contrast, stage 1 OCs, which are confined to the ovary, have a survival rate approaching 95% [1]. As evidenced by this, improving OC survival will require a better understanding of early stages of the disease, as well as the mechanisms by which OCs become invasive and metastatic.

Studies investigating hereditary OC risk and early stage disease have determined that the majority of hereditary OCs are associated with mutation of the BRCA1 gene [2]. Carriers of a BRCA1 mutation have a 30% – 40% risk of developing OC during their lifetime [3, 4]. The gene product of the BRCA1 gene is the multifunctional tumor suppressor BRCA1, which is involved in cell cycle, DNA damage response, chromatin remodeling [5], and ubiquitin ligase activity [6]. Founder mutations of BRCA1 occur at a high frequency in genetically isolated populations, such as the Ashkenazi Jewish population. The 185delAG founder mutation, BRAT, is the deletion of two nucleotides
(AG) in the second exon of the BRCA1 gene. This deletion results in a reading frame shift and likely results in translation of a premature stop signal at codon 39 and a truncated protein product. Though the BRAT mutation is detected in clinical samples and solidly correlates with OC risk, cross-reactivity of antibodies with wt BRCA1 has made detection of the truncated protein in clinical samples unsuccessful. However, recent studies suggest that mutant BRCA1 proteins may contribute to cancer by unique gain-of-function activities, independent of the loss of normal BRCA1 function, by influencing proliferation, apoptosis, and gene regulation (Reviewed in [7]). For example, we have previously found that expression of BRAT in normal HOSE cells increases expression of the tumor suppressor maspin, which is uniquely up-regulated in OC [8, 9].

Preliminary microarray data reveal an additional downstream target of BRAT, the matrix metalloprotease 1 gene. MMP1 is a member of the matrix metalloprotease family of enzymes, which utilize Zinc at the active site to cleave a broad spectrum of substrates, including ECM constituents, pro-growth factors, growth factor receptors, and cell-cell adhesion mediators [10]. MMPs are secreted as zymogens and activated upon cleavage and removal of their auto-inhibitory pro domain by other MMPs or serine proteases [11]. Specifically, MMP1, also known as interstitial collagenase I, is capable of cleaving several substrates of potential importance for OC, including collagens I and III. In vitro, MMP1 also cleaves fibronectin, and laminin, which are present in the ovarian and omental extracellular matrix, as well as pro-MMP2 and 9 and pro-TNFα [12-14].

In the normal ovary, MMPs are up-regulated in the pre-ovulatory follicle [15] and may be secreted by HOSE cells from lysosomes [16] to aid in release of the oocyte during ovulation. MMP mutations and gene amplifications are not generally reported, but
MMP expression is increased in multiple cancer types, including ovarian [11]. For example, Kenny et al. demonstrate that adhesion of SkOV3 OC cells to mouse peritoneum decreases after MMP2 inhibition or knock down, and that MMP2 expression is enhanced in adherent cells [12]. Agarwal et al. implicate MMP1 in the activation of protease activated receptor 1 (PAR-1) and demonstrate the importance of this pathway for OC ascites formation, metastasis, and angiogenesis [17].

In this chapter, I demonstrate that the BRAT mutation increases MMP1 expression and pro-MMP1 secretion by HOSE cells, and that this up-regulation occurs through c-Jun and specific AP-1 sites in the MMP1 promoter. Further, I demonstrate that immortalized HOSE derived from BRAT mutation carriers also express significantly elevated levels of pro-MMP1. In accordance with these data, I hypothesize that enhanced MMP1 expression and secretion by the OSE of patients carrying the BRAT mutation may represent an initial step toward cellular malignancy and ultimately mutation-associated OC initiation and progression.

**Methods**

**Cell culture and transfection.** The SV 40-Large T-Ag transfected HOSE cells: wt BRCA1 confirmed: (HOSE-118 [18]), negative family history of OC: HOSE-121, IMCC5, and 185delAG confirmed: (3261-77a, 3261-77b [19], 1816-686a, and 1816-686b [20]) were cultured in Medium 199/ MCDB 105 (Sigma, St. Louis, MO) with 10% fetal bovine serum (FBS) and gentamicin. Multiple stable BRAT clones were generated by transfection of 2-3 million HOSE 118 cells with 2-2.5ug of PCDNA3.1 or Flag-BRAT [18]. Stable lines were maintained in 1 mg/ml G418 selection media and confirmed to express BRAT by RT-PCR [18]. All cells were incubated at 37°C with 5% CO₂. Two and
a half million cells were transiently transfected as previously described [18] using Program X-005, Kit V, and the Nucleofector device (Amaxis/ Lonza, Walkersville, MD) with 1.5-2.5 ug of plasmid (BRIT, Flag-BRAT [18], pGL4.74 Renilla Luciferase/TK (Promega, Madison, WI), human MMP1 luciferase reporters (1G and 2G wt (full length), 1G and 2G (-3292), 1G and 2G (-2942), (-1546), and (-517) [21], 1G and 2G (Δ1602), 1G and 2G (Δ72) [22], or green fluorescent protein (GFP). The control plasmid BRIT was generated by inserting a frameshift mutation to abrogate the BRAT sequence from amino acids 22-38 in the BRAT plasmid, and has been previously characterized as an appropriate control [18]. The full length MMP1 promoter consists of the 4372 base pairs directly upstream of MMP1 gene start codon, and extends to +63. The reporter plasmids in the truncation series are truncated from the 5’ end of the promoter at the indicated base pair. The Δ1602 and Δ72 constructs represent the 1G and 2G versions (at -1607) of the full length promoter in which the AP1 site at the indicated location has been mutated and rendered non-functional. For knockdown studies, cells were co-transfected with 2ug Smartpool small interfering RNA (siRNA) targeting c-Jun (Si c-Jun) or Smartpool non-targeting control siRNA (Si Con) from Dharmacon (Chicago, IL).

**Microarray.** HOSE-118 cells were serum-starved for 24 hours (h), and transfected with 3ug of indicated transfectant (PCDNA3.1 or flag-BRAT) using Lipofectamine reagent (Life Technologies, Inc., Grand Island, NY) [23]. Twenty-four hours after transfection, cells were collected in Trizol reagent (Invitrogen, Carlsbad, CA). Total RNA was purified using the RNeasy cleanup procedure (Qiagen Inc., Valencia, CA). The quality of total RNA was assessed by agarose gel electrophoresis and A260/A280 ratio or by analysis on the Agilent 2100 Bioanalyzer.
Five micrograms of total RNA from each sample was processed for microarray analysis. The poly(A) RNA was specifically converted to complementary DNA (cDNA) and then amplified and labeled with biotin following the procedure initially described by Van Gelder et al. [24]. Hybridization with the biotin labeled RNA, staining, and scanning of the chips followed the prescribed procedure outlined in the Affymetrix technical manual and has been previously described [25]. Probe arrays: The oligonucleotide probe arrays were the Affymetrix U133A human arrays. These arrays consist of 22,215 probe sets, which target known and suspected genes as well as a number of suspected splice variants. Data Analysis: Scanned output files were visually inspected for hybridization artifacts and then analyzed using Affymetrix Microarray 5.0 software. Signal intensity was scaled to a trimmed mean intensity of 500 prior to output. For direct comparison analysis the MAS 5.0 software was used to identify differentially expressed genes between paired samples. The Wilcoxon’s one-sided signed rank test was used to assess the behavior of all oligonucleotide probes in each probeset [26]. Probe sets that yielded a change p-value less than 0.005 were identified as changed (increased or decreased). The analyses of replicate experimental pairs were intersected to produce the final list. Using the MAS 5.0 calculated signal value the list was further reduced by removing probesets with low expression value in all samples and where the average change in signal value between the control condition and the experimental condition was less than 1.4-fold.

**Western blot.** Cells were washed in phosphate buffered saline (PBS), trypsinized, pelleted, and washed 1-2 times in cold PBS. Cells were lysed for 30 minutes on ice in modified CHAPS buffer, and lysate was centrifuged at 115,000 xg, at 4 degrees C, for 1h.
Thirty to 100μg of protein were separated via 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to Polyvinylidene fluoride (PVDF) membranes, dried, and blocked in 5% milk in Tween 20-Tris buffered Saline. Blots were incubated in their respective antibodies overnight, followed by incubation with a horseradish peroxidase (HRP)-conjugated secondary (Fisher, Pittsburgh, PA), and developed via enhanced chemiluminescence substrate (ECL) (Pierce/ Fisher, Pittsburgh, PA). Antibodies: c-Jun (1:1000) Cat. #9165, Phospho-c-Jun (1:500) Cat. #9261, Cell Signaling Technology (Beverly, MA), Actin clone AC-40 (1:10,000) Cat. #4700 Sigma (St. Louis, MO), MMP1 (2ug/mL) Cat. #MAB901 R&D Systems, Inc. (Minneapolis, MN), Ets-1 (1:500) Cat. #sc-56674 Santa Cruz (Santa Cruz, CA).

**RT-PCR.** RNA samples were isolated using TRIzol reagent from Invitrogen (Carlsbad, CA) per manufacturers protocol and DNAse treated.

For semi-quantitative PCR, 1 microgram total RNA, oligo(dT), and reverse transcriptase were used to generate single-strand cDNA as previously described [18]. The cDNA samples were amplified using the Applied Biosystems GeneAmp RNA PCR Core Kit (Foster City, CA). Primers used were: Flag-BRAT sense (CGATGACAAAATGGATTTATCTGC), Flag-BRAT antisense (GAGACAGGTTCTTCTTACTCAACTCC), MMP1 sense (GAGCAAAAACATCTGAGGTACAGGA), MMP1 antisense (TTGTCCCGATGATCTCCCTGACA) [27], actin (98 base pairs) sense (GGGAATTCAAAACTGGAACGGTGAAGG), and actin (98 base pairs) antisense (GGAAGCTTATCAAAGTGTCCTCGGCCACA). The amplified products were separated
by electrophoresis on a 10% polyacrylamide gel, stained with SYBR Green (Lonza, Rockland, ME), and photographed with the Kodak EDS 120 Digital Analysis System. The net intensity of each band was normalized to the respective endogenous control band.

For real time PCR, 100 ng total RNA was reverse transcribed to generate single-strand cDNA as previously described [18]. The cDNA samples were amplified using Fast SYBR Green Master Mix (Applied Biosystems) on an Applied Biosystems Step One Plus instrument. RQ (relative mean mRNA expression level) is calculated by the Step One software version 2.0. Using standard curves constructed for target and endogenous control genes, an arbitrary quantitative gene expression value is determined from the threshold cycle (Ct) for each gene for each sample. Target gene values are normalized to control gene values, and fold difference is determined by dividing by the designated reference/calibrator sample.

**Enzyme-linked immunosorbant assay.** For conditioned media analysis, media containing 0.1% FBS was added to cells 24 h after transfection/plating. Twenty-four hours later, cells were counted and media was collected and centrifuged to remove debris, aliquoted, and stored at (-80)°C. To assess MMP1 activity, MMP1 Enzyme Linked Immunosorbant Assay (ELISA) Enzyme Activity Assay (R&D Systems, Inc, Minneapolis, MN) was performed on un-concentrated conditioned media samples in triplicate according to manufacturer’s protocol. Total MMP1 (pro and active forms) was detectable by addition of APMA MMP activating agent to all sample wells and standards. Fluorescence was read on a Fluorostar Galaxy plate reader. Resultant values were derived from a standard curve and expressed as the mean MMP1 concentration of triplicate samples +/- standard error. When cell viability varied significantly, MMP1 concentration
was normalized to average cell number at time of conditioned media collection. MMP2 and 3 ELISAs (R&D Systems, Inc, Minneapolis, MN) were performed in triplicate on unconcentrated conditioned media as per manufacturer’s protocol.

**Luciferase assay.** Stable 118 PCDNA3.1 or BRAT cells were transfected with 0.15ug Renilla Luciferase reporter and 1.5ug MMP1 luciferase reporter. Twenty-four hours later, cells were collected in Promega Passive Lysis Buffer and subjected to 2 freeze-thaw cycles. Lysates were centrifuged at 10,600 xg for 1 minute at 4 degrees C, and supernatant was collected. Luciferase activity was assessed using a manual luminometer and the Promega Dual Luciferase Assay System according to manufacturer protocol. For knockdown reporter studies, siRNA was co-transfected and cells were collected 48 hours after transfection.

**Statistics.** For real time PCR, error bars illustrate RQmin and RQmax, which are calculated as: 
\[
RQ_{ave} \text{ divided by (standard deviation}^{\text{ student’s t value at the 95% confidence interval, for 5 degrees freedom}}) \text{ and } RQ_{ave} \text{ times (standard deviation}^{\text{ student’s t value at the 95% confidence interval, for 5 degrees of freedom}}, \text{ respectively. This range represents the confidence interval at the 95% confidence level. For ELISA data, student’s t test was performed to assess statistical difference between means of replicates. For ELISA comparison of MMP1 in conditioned media in cell lines, data did not follow a normal distribution; therefore, a nonparametric Wilcoxon test was performed.**

**Results**

**BRAT alters expression of genes involved in multiple cellular processes.**

Several studies supporting the importance of independent BRCA1 mutant functions
report modulation of gene expression by mutants. The periostin gene was up-regulated in HeLa cells stably expressing the risk-associated BRCA1 mutation 5083del19, which lacks the C terminal 193 amino acids, but not wt BRCA1, while increased periostin levels were also found in breast cancer tissue and serum of patients carrying the mutation [28]. Similarly, mRNA and protein of D52 (TD52) and the folate receptor alpha (FOL1) were elevated in Hela cells expressing the Ser1841Asn breast cancer risk-associated BRCA1 mutation [29], and a synthetic BRCA1 truncation mutant comprising the N-terminal third of the protein up-regulated p53 gene expression and down-regulated constituents of the JNK/SAPK and MAPK/ERK pathways in mouse epithelial OC cells [30]. Preliminary microarray analysis of HOSE 118 cells (wt BRCA1), transiently transfected with the BRAT mutation revealed numerous differentially regulated genes (Table 2.1.), including elevated expression of the gene encoding matrix metalloprotease 1.

**BRAT enhances MMP1 gene expression in HOSE 118 cells.** MMP family members are important for invasion and metastasis and are overexpressed in OC [11]. In addition to MMP1’s role in PAR-1 activation in OC [17], a polymorphism in the MMP1 promoter that enhances expression [22] was found more frequently in OC patients compared to normal women. Further, tumors with the allele expressed seven times more MMP1 than tumors without the allele [31]. Therefore, I decided to further explore MMP1 as a downstream target of BRAT in HOSE cells.

To confirm that BRAT enhances MMP1 expression transcriptionally, I performed semi-quantitative RT-PCR to measure the MMP1 mRNA level in HOSE 118 cells stably expressing BRAT or the control vector PCDNA3.1. While vector-expressing cells exhibited undetectable levels of MMP1 by this method, MMP1 mRNA levels were
Table 2.1. Selection of genes determined to be differentially regulated in BRAT cells.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Change PCDNA3.1 vs BRAT</th>
<th>Average fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL1 alpha</td>
<td>I</td>
<td>4.61</td>
</tr>
<tr>
<td>BCL2A1</td>
<td>I</td>
<td>4.55</td>
</tr>
<tr>
<td>IL1 beta</td>
<td>I</td>
<td>3.84</td>
</tr>
<tr>
<td>NFkB2</td>
<td>I</td>
<td>3.60</td>
</tr>
<tr>
<td>MMP1</td>
<td>I</td>
<td>2.70</td>
</tr>
<tr>
<td>IL6</td>
<td>I</td>
<td>2.50</td>
</tr>
<tr>
<td>IL11</td>
<td>I</td>
<td>2.06</td>
</tr>
<tr>
<td>Collagen I alpha 1</td>
<td>D</td>
<td>-1.84</td>
</tr>
<tr>
<td>Collagen III alpha 1</td>
<td>D</td>
<td>-2.65</td>
</tr>
</tbody>
</table>
significantly enhanced in both BRAT clones 6 and 7 (Figure 2.1. A). MMP1 mRNA levels were also evaluated by real time RT-PCR, and BRAT cells exhibited MMP1 mRNA levels 16-18 fold higher than PCDNA3.1 cells (Figure 2.1. B). This data supports the hypothesis that BRAT enhances transcription of the MMP1 gene in HOSE 118 cells.

**BRAT increases expression and secretion of pro-MMP1 by HOSE 118 cells.**

To evaluate the impact of BRAT expression on intracellular protein levels of MMP-1, Western blotting was performed on lysates of stable HOSE 118 BRAT and PCDNA3.1 cells. As expected, active (cleaved) MMP-1 was not detectable in lysates, however levels of pro-MMP1 were 5-21-fold higher in BRAT clones 6 and 7 compared to PCDNA3.1 cells (Figure 2.2. A.). To determine whether BRAT expression also results in increased pro-MMP1 protein secretion, the MMP1 Enzyme Activity Assay was utilized to measure enzyme activity in conditioned media of cultured cells. Addition of a chemical MMP activating reagent, 4-Aminophenylmercuric acetate (APMA), allows measurement of total MMP1 (pro and active). Endogenously activated MMP1 was not detectable in un-concentrated conditioned media of HOSE 118 cells, however, total MMP1 levels were 6-8 fold higher in conditioned media of BRAT clones 6 and 7 compared to PCDNA3.1 cells (Figure 2.2. B). BRAT-mediated up-regulation was specific to MMP1, as MMP2 and 3 were undetectable in conditioned media of transiently transfected BRIT or BRAT cells (Figure 2.2. C). These data indicate that BRAT specifically enhances expression and secretion of pro-MMP1 by HOSE 118 cells.

**MMP1 and maspin are independent targets of BRAT.** As mentioned previously, maspin was determined to be up-regulated in BRAT-expressing HOSE cells [8]. Maspin is a member of the serine protease inhibitor (serpin) family that was
Figure 2.1. MMP1 mRNA is increased in BRAT-expressing HOSE cells. Cells stably expressing BRAT or the PCDNA vector were plated at equal densities and collected. RNA was isolated, DNAse treated, and reverse transcribed. A. Semi-quantitative PCR was performed for MMP1 and actin. PCR products were electrophoresed on a 10% acrylimide gel, stained with SYBR green, and imaged. B. Real time PCR was performed in triplicate for MMP1 and actin using SYBR green detection. RQ (relative mean mRNA expression level) was calculated by the Step One software version 2.0. Using standard curves constructed for target and endogenous control genes, an arbitrary quantitative gene expression value is determined from the Ct for each gene for each sample. Target gene values are normalized to control gene values, and fold difference is determined by dividing by the designated reference/calibrator sample.
A.

<table>
<thead>
<tr>
<th>PCDNA PCDNA</th>
<th>BRAT6</th>
<th>BRAT6</th>
<th>BRAT7</th>
<th>BRAT7</th>
<th>BRAT7</th>
</tr>
</thead>
</table>

Pro-MMP1

| Actin |

Relative Band Intensity

Lane

1 2 3 4 5 6 7 8

0 0.5 1 1.5 2 2.5 3

B.

Cell Type

PCND3.A3.1 BRAT6 BRAT7 BRAT7

Total MMP1 ng/mL

p < 0.001 p < 0.001 p = 0.001
Figure 2.2. BRAT increases cellular pro-MMP1 and total secreted MMP1 in BRAT cells. A. Cells stably expressing PCDNA or BRAT were plated at equal densities, collected, and lysed. Protein lysate was separated using SDS-PAGE and Western blotting was performed using the indicated antibodies. Relative band intensity was determined by dividing the pro-MMP1 band intensity by the actin band intensity for each lane. B. Stable cells were plated at equal densities, allowed to attach, and then serum starved in media containing 0.1% fetal bovine serum for 24 hours. Conditioned media was collected and debris and dead cells were removed by centrifugation. Total MMP1 levels in unconcentrated conditioned media were determined in triplicate by an MMP1 ELISA activity assay using the addition of the MMP activating reagent APMA. Graph indicates averages +/- standard error. C. Cells were transiently transfected with indicated transfectant and conditioned media was collected as described. Total MMP levels in unconcentrated conditioned media were determined in triplicate by ELISA kits. Graphs illustrate averages +/- standard error, except positive control human dermal fibroblast and human umbilical vein endothelial cell (HUVEC) conditioned media.
identified by its diminished expression in breast tumor samples compared to normal breast tissue [32]. Maspin is non-inhibitory due to a shortened and non-conserved reactive site loop, the serpin domain responsible for protease inhibition [32]. Maspin expression is low or absent in normal HOSE [9, 33], but in OC samples correlates with high tumor grade and shorter overall survival [9]. Exogenous maspin expression inhibits migration, angiogenesis, invasion, and metastasis in multiple cancer models in vitro and in vivo, including breast, prostate, and OC cells [9, 34-36]. Because maspin has been identified as a target of BRAT and maspin modulates migration, invasion, and metastasis, I decided to test whether maspin impacts MMP1 expression in BRAT cells. MMP1 mRNA levels were not significantly impacted by maspin knockdown (Figure 2.3), suggesting maspin and MMP1 are parallel targets of BRAT.

**BRAT-mediated MMP1 modulation is c-Jun dependent.** MMPs are regulated extracellularly by pericellular localization [11], proteolytic activation, and binding of tissue inhibitors of metalloproteases (TIMPS) [37], as well as intracellularly by transcription. To begin to elucidate the mechanism of MMP1 gene up-regulation in BRAT cells, I chose to evaluate the importance of c-Jun, as this transcription factor was previously implicated in BRAT-mediated maspin up-regulation in HOSE 118 cells [8], and because multiple AP1 sites in the MMP1 promoter are important in gene regulation (Reviewed in [38-40]). HOSE 118 cells were transiently co-transfected with BRIT or BRAT and Si Con or Si c-Jun. BRIT/BRAT expression was confirmed by semi-quantitative PCR (Figure 2.4. A). Greater than 95% knockdown of c-Jun was confirmed by Western Blot (Inset in Figure 2.4. B). c-Jun knockdown diminished MMP1 mRNA levels by 60% in BRIT cells, and by 80% in BRAT cells (Figure 2.4. B). Further, c-Jun
Figure 2.3. MMP1 and maspin are parallel targets of BRAT. HOSE 118 cells stably expressing PCDNA or BRAT were transiently transfected with siRNA targeting maspin. Cells were collected after 48 hours, and RNA was isolated, DNase treated, reverse transcribed, and real time PCR was performed in triplicate for MMP1 and actin using SYBR green detection. Semi-quantitative PCR was performed for maspin and actin, and PCR products were run on a 10% acrylimide gel, stained with SYBR green, and imaged.
**Figure 2.4.** BRAT-mediated MMP1 modulation is c-Jun dependent.

A. 118 HOSE cells were transiently transfected with indicated transfectant and cells were collected 48 hours after transfection. RNA was isolated, DNase treated, reverse transcribed, and semi-quantitative PCR was performed for BRIT/BRAT and actin. PCR products were electrophoresed on a 10% acrylimide gel, stained with SYBR green, and imaged. B. Protein lysates were collected in parallel for knock-down analysis by Western blot. Real time PCR was performed in triplicate for MMP1 and actin using SYBR green detection. C. Conditioned media was collected in parallel. MMP1 ELISA activity assay was performed in triplicate as described, however, because cell viability differed significantly, cell counts were performed upon conditioned media collection, and total MMP1 levels were normalized to cell number. Graph illustrates averages +/- standard error.
knockdown also reduced total MMP1 level in conditioned media by 69% in PCDNA cells, and 81% in BRAT cells (Figure 2.4. C). Interestingly, c-Jun protein levels were elevated in BRAT cells (1.4 fold) (Figure 2.4. B), suggesting that BRAT-mediated MMP1 up-regulation may occur at least, in part, through increased c-Jun levels.

**AP-1 sites in the MMP1 promoter mediate enhanced MMP1 expression in BRAT cells.** Specific AP1 sites in the MMP1 promoter have been shown to impact MMP1 gene expression. The AP1 site at (-72) is important for basal as well as induced MMP1 expression [41, 42]. In addition, an AP1 site at (-1602) is important for regulation, and this region is of critical importance in OC. A single nucleotide polymorphism in the MMP1 promoter, the insertion of an extra “G” nucleotide at (-1607), designated “2G”, was found to increase the efficacy of an Ets transcription factor binding site adjacent to the AP1 site at (-1602), and increase MMP1 expression in normal human fibroblasts and melanoma cells [22]. Interestingly, compared to the 1G allele, the 2G allele was present in a higher proportion of OC patients compared to normal controls [31]. To determine whether BRAT mediates 1G/2G allele-specific regulation of the MMP1 promoter, a set of MMP1 luciferase reporter constructs was obtained (Figure 2.5. A). Each construct contains the full length MMP1 promoter with either the 1G or 2G allele at (-1607) in control of the firefly luciferase gene.

Activity of the 1G wt reporter was 1.7 fold higher in stable BRAT cells compared to stable PDCNA cells, while activity of the 2G wt reporter was greater than 2 fold higher in BRAT cells (Figure 2.5. B). Further, the 2G wt reporter construct mediated significantly more activity than the 1G promoter in both PCDNA (3.4-fold) and BRAT cells (4.4-fold). The importance of the (-72) and (-1602) AP1 sites was also determined
A.

1G

5' AAGAT 3'

(-1607)

2G

5' AAGGAT 3'

(-1607)

Full length wt MMP1 promoter (-4372) to (+63): 1G and 2G Versions

**1G**

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Red crosses indicate deletions.
**Full Length (-4372):** All transcription factor binding sites intact

(-3292): Loss of OCTA3, Putative silencers (x4), PEA-3, AP-1

(-2942): Loss of CREB, PEA-3

(-1546): Loss of Dorsal, AP-2, Putative anti-silencers (x3), CBP (x2),
ATF/CREB, Putative Silencers (x3), SP-1, PEA3, c-fos-US5,
AP-1 (x2), CACCC box (x2)

(-517): Loss of AP-1 (x3), CACCC box, TTCA motif, PEA-3 (x2), TATA Box

**B.**

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<thead>
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<th>PCDNA3.1</th>
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<td><strong>p&lt;0.0006</strong></td>
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**Ave Luciferase Activity**

![Graph showing luciferase activity](image)

- **p<0.0005**
- **p<0.0006**
- **#**
- ***"**
Figure 2.5. AP-1 sites in the MMP1 promoter mediate enhanced MMP1 expression in BRAT cells.

A. Human MMP1 promoter luciferase reporter constructs. B. Cells stably expressing PCDNA or BRAT were transiently transfected with the indicated MMP1 reporter construct and a Renilla constitutive luciferase reporter plasmid for normalization. Lysates were collected, subjected to two freeze-thaw cycles, and assayed in triplicate on a manual luminometer using Promega’s Dual Luciferase Assay Kit. Luciferase activity was normalized to Renilla luciferase activity for each triplicate, averaged, and results are expressed +/- standard error. C. Stable cells were also co-transfected with non-targeting control siRNA or siRNA targeting c-Jun, collected, and assayed similarly. Protein lysates were collected in parallel for knock-down analysis.
by utilizing 1G and 2G full length promoter constructs in which the indicated AP1 site, (-72) or (-1602), was mutated to be non-functional (Figure 2.5. A). In reporter constructs containing either 1G or 2G version of the SNP, MMP1 promoter activity was diminished significantly by mutation of either AP1 site, (-72) or (-1602) (Figure 2.5. B). These data suggest that AP1 sites at (-72) and (-1602) are critical for BRAT-mediated enhancement of MMP1 expression. Further, the presence of an Ets binding site adjacent to the distal AP1 site further augments c-Jun-mediated MMP1 transcription.

To dissect the contribution of other regions of the promoter to BRAT-mediated MMP1 up-regulation, a second set of luciferase reporter constructs was utilized. The constructs consist of a series of 5’ truncations of the MMP1 promoter in control of the firefly luciferase gene (Figure 2.5. A). The full length (FL), (-3292), and (-2942) constructs have the 2G version of the SNP, while the smaller constructs, (-1546) and (-517) lack this region. SiRNA against c-Jun was used concomitantly to validate the importance of c-Jun for BRAT-mediated MMP1 promoter activity mediated by each construct. Activity of the (-3292) truncation, in which an OCTA3 site, a PEA3 site, an AP1 site, and several silencers are lost, was decreased by 24% compared to the full length reporter (Figure 2.5. C). Truncation to (-2942) results in loss of cAMP response element binding (CREB) and polyoma enhancer activator protein (PEA3) sites from the promoter. Transfection of (-2942) yielded 18% lower activity than the full length promoter as well. Notably, c-Jun knockdown in BRAT cells reduced full length, (-3292), and (-2942) reporter activity 61, 62, and 66.5% respectively, confirming the importance of c-Jun for BRAT-mediated MMP1 expression. Similarly, c-Jun knockdown significantly diminished remaining activity of the (-1546) and (-517) constructs. These
data suggest that proximal portions of the MMP1 promoter are important for BRAT-mediated up-regulation, and that AP1 sites throughout the promoter are necessary for MMP1 expression in BRAT cells.

**Increased pro-MMP is detectable in BRAT mutation carrier-derived cellular conditioned media.** To evaluate the clinical impact of BRAT on MMP1 levels, BRAT mutation carrier-derived immortalized HOSE cell lines were utilized. MMP1 levels in conditioned media of immortalized HOSE cell lines from the ovaries of women carrying a BRAT mutation were compared to lines without a family history of OC or with a confirmed wt genotype. Total conditioned media MMP1 levels were significantly higher in BRAT carrier HOSE cell lines (3261-77a, 3261-77b, 1816-686a, and 1816-686b) compared to non-carrier HOSE cell lines (121, 118, IMCC5) (Figure 2.6.). These data suggest that BRAT-mediated MMP1 up-regulation is detectable in cells of patients carrying this mutation.

**Discussion**

We have previously shown a role for the 185delAG BRCA1 mutation, BRAT, in STS-induced apoptosis of normal HOSE and OC cells [18], as well as in the up-regulation of the OC-associated serpin, maspin, in normal cells [8]. Here, I identify a novel downstream target of BRAT, MMP1, and show that MMP1 is up-regulated transcriptionally through a mechanism involving c-Jun. Further, ELISA reveals higher total MMP1 secretion by HOSE cell lines derived from BRAT mutation carriers.

Interestingly, several of the genes differentially regulated in BRAT-expressing HOSE cells encode proteins that localize to the extracellular space and are potentially important in OC (collagen I, and collagen III, IL-6, IL-1alpha, IL-1beta, and MMP1).
Figure 2.6. Increased pro-MMP is detectable in BRAT mutation carrier-derived cellular conditioned media.
Cells were plated in triplicate at similar densities and conditioned media was collected as described. MMP1 ELISA activity assay was performed in triplicate as described, however, because cell viability differed significantly, cell counts were performed upon conditioned media collection, and total MMP1 levels were normalized to cell number. Graph illustrates averages +/- standard error.
Collagens I and III are present in the extracellular matrix of the ovary and/or the omentum [12, 13]. IL-1β enhances MMP1 mRNA stability and expression [43] and up-regulates tumor necrosis factor α (TNFα) [44], another potential BRAT target and mitogenic factor for HOSE cells [14], while IL-6 can enhance invasion of OC cell lines [45]. MMP1 cleaves collagens I and III and can also cleave pro-TNFα, fibronectin, and laminin, which are present in the ovarian and omental ECM, and pro-MMP 2 and 9, which cleave multiple extracellular matrix constituents [11]. Taken together, these targets of BRAT could promote motility, invasion, and metastasis of normal HOSE and potential tumor cells of mutation carriers.

C-Jun’s importance in MMP1 gene regulation has been well established [41, 42], and in my model system, C-Jun is crucial for BRAT-mediated MMP1 up-regulation. Increased C-Jun protein levels in BRAT cells likely contribute to MMP1 up-regulation. In agreement, C-Jun mRNA levels increase significantly prior to TNFα and IL-1-induced MMP1 up-regulation [46]. Future studies may reveal additional C-Jun-responsive genes differentially regulated by BRAT.

I have begun to elucidate specific AP1 sites and other MMP1 promoter elements necessary for BRAT-mediated MMP1 up-regulation. Truncation of the full length MMP1 promoter to (-3292) significantly decreases reporter activity in BRAT cells, which indicates the importance of one or more distal promoter elements for activity. Indeed, binding sites for AP1 and PEA3, which has been shown to transactivate the MMP1 promoter [47], are located here. In some experiments, activity of the (-2942) construct was marginally higher. Loss of a PEA3 site at this position could make available additional PEA3, which has been shown to synergize with C-Jun [47]. BRAT cell reporter
activity of the three largest constructs was dramatically decreased by c-Jun knockdown. Reduced activity was comparable to that of the full length promoter in PCDNA3.1 cells, confirming the importance of c-Jun for BRAT-mediated MMP1 expression. The greatest loss in reporter activity relative to full length occurs by truncation of the promoter to (-1546) or (-517), by which several elements are eliminated, including two AP1 sites. Specific disruption of the (-1602) AP1 site significantly decreases reporter activity as well. In agreement with the literature (Reviewed in [38]), one or more of the most proximal AP1 sites (-1062, -891, -562, -436, -181, -72) contribute to basal BRAT-mediated MMP1 promoter activity, as c-Jun knockdown significantly diminishes remaining activity of the (-1546) and (-517) constructs. Further, specific disruption of the (-72) AP1 site also abrogates reporter activity. Taken together, these data reveal the necessity of c-Jun in BRAT-mediated MMP1 up-regulation.

The presence of the 2G MMP1 promoter polymorphism increases promoter activity [22] and is associated with decreased disease-free and overall survival in OC patients [48]. As expected, BRAT cells exhibited significantly higher activity than PCDNA3.1 cells when measuring 1G and 2G versions of the promoter. In agreement with previous findings, the 2G wt reporter construct mediated significantly more activity than the 1G promoter in both PCDNA3.1 and BRAT cells. Interestingly, the enhancement in promoter activity mediated by the additional G nucleotide was even more apparent in BRAT cells, and Ets-1 levels were increased in BRAT cells compared to PCDNA cells (Figure 2.7.). Greater MMP1 2G promoter activity in BRAT cells may occur through cooperation of Ets-1 with increased c-Jun, as Ets-1 and c-Jun physically interact and can synergistically transactivate promoter expression [49]. Further, AP1 and
Figure 2.7. Ets-1 protein levels are elevated in BRAT cells. Cells stably expressing PCDNA or BRAT were plated at equal densities, collected, and lysed. Protein lysate was separated using SDS-PAGE and Western blotting was performed using the indicated antibodies. Relative band intensity was determined by dividing the Ets-1 band intensity by the actin band intensity for each lane.
Ets-1 sites are found in the Ets-1 promoter [50], therefore increased Ets-1 levels in BRAT cells may occur in part through increased c-Jun transactivation. Alternatively, Ets-1 may represent an additional independent signaling pathway alternatively regulated in BRAT cell. It would be interesting to determine whether BRAT mutation-associated OCs exhibit a high frequency of 2G alleles, as the combination of this allele with elevated c-Jun protein could potentially augment OC progression or metastasis by up-regulating MMP1.

I have determined that maspin and MMP1 are parallel targets of BRAT, as maspin does not significantly impact MMP1 expression in BRAT cells. Interestingly, MMP1 is activated in vitro in a stepwise manner by uPA and MMP3 (stromelysin) [51], and maspin has been shown to negatively regulate uPA activity by enhancing uPA/uPAR internalization and by inhibiting activation of pro-uPA [52]. Though maspin doesn’t appear to regulate MMP1 expression, it may be important in regulation of MMP1 activity. Indeed, DU145 prostate cancer cells stably expressing maspin exhibit decreased collagen cleavage in collagen-degradation assays [53].

We have previously found significantly lower levels of phospho-Akt concurrent with enhanced apoptosis in BRAT cells treated with cytotoxic drugs [18]. It would be interesting to determine whether diminished Akt activity is necessary for MMP1 up-regulation in BRAT cells, and the signaling pathways involved. Indeed, preliminary data suggest Akt signaling is involved in BRAT-mediated MMP1 up-regulation. Expression of a constitutively active Akt construct diminishes MMP1 mRNA expression and total MMP1 in conditioned media of BRAT cells (Figure 2.8.). Interestingly, Akt inhibition has been shown to enhance activation of the MAPK pathway [54], which in turn
Figure 2.8. Constitutively active Akt reverses BRAT-mediated MMP1 up-regulation. 
A. 118 HOSE cells were transiently transfected with indicated transfectant and collected 24 hours later. RNA was isolated, DNAs treated, reverse transcribed, and real time PCR was performed in triplicate for MMP1 and actin using SYBR green detection. Protein lysates were collected in parallel for analysis of CA-Akt expression. B. Conditioned media was collected in parallel. MMP1 ELISA activity assay was performed in triplicate as described, however, because cell viability differed significantly, cell counts were performed upon conditioned media collection, and total MMP1 levels were normalized to cell number. Graph illustrates averages +/- standard error.
enhances activity of the transcription factor c-Jun. It will be interesting to determine in
future studies the mechanism by which Akt modulates MMP1 expression.

Normal cell lines and prophylactically removed ovaries from BRCA1 mutation
carriers provide model systems in which to study early genetic, cellular, and histologic
steps in OC initiation and progression. Immunohistochemical analysis revealed
intermediate levels of Ki67 and p53 staining for prophylactically removed ovaries, while
normal ovaries had the lowest staining and OC specimens exhibited the highest staining
[55]. Not all of the prophylactically removed ovaries were from patients with confirmed
BRCA1 mutations, however, and no correlation was found between any of the markers
and presence of a BRCA1 mutation [55]. In contrast, Piek et al. found no histologic
changes in prophylactically removed ovaries compared to normal ovaries, and no
difference in staining of Ki67, p21, p27, p53, Cyclin A, cyclin D1, Her2, ERalpha, and
PR. Only Bcl-2 expression was significantly higher than in normal ovaries [56].

Kirkpatrick et al. devised a multiphoton microscopy method to image ovarian
surface epithelium and collagen fibrils of the ECM immediately after oophorectomy.
Changes in collagen fibril organization similar to cancer specimens were detected in high
risk ovaries [57]. Similarly, abnormal regions of OSE, such as invaginations, inclusions
cysts, papillary and stratified areas, adenomas, and microscopic adenocarcinomas, lacked
a detectable basement membrane, as defined by collagen IV and laminin staining [58].
These studies suggest that alteration in the ECM integrity may be a characteristic of
BRCA1 mutant OC pathology.

Early molecular and cytological changes have also been seen in cell lines
generated from normal OSE cells of women with a strong family history of OC,
including irregular cellular morphology and organization in culture, elevated CA-125 and E-cadherin, and amplification of various signaling pathways such as hepatocyte growth factor (HGF) and the PI3K pathway (Reviewed in [59]). Immortalized HOSE from these patients also exhibited higher telomeric instability when compared to cells from patients without a strong family history [20].

Our investigation reveals BRAT mutation-associated phenotypic changes, as mutation carrier-derived cell lines exhibit elevated MMP1 secretion. Cleavage of ECM components, cell-cell adhesion molecules, growth factors/receptors, and other proteases is a crucial step in cancer cell migration, invasion, and metastasis. Consequently, up-regulation and enhanced secretion of pro-MMP1 from the HOSE of BRAT mutation carriers could prime these cells for transformation and metastasis. Cleavage of pro growth factors and release of ECM-bound growth factors potentially promote survival and, therefore, accumulation of cancer-promoting mutations. ECM remodeling also alters cell-ECM contacts, which influence cell survival and migration as well. Finally, as mentioned previously, OCs are highly prone to metastasis through cell shedding, and MMPs are critical for OC cell attachment at metastatic sites and establishment of metastases [12]. Though MMP inhibitors have been largely ineffective in clinical trials, this may be in part because they are administered after metastases are already established [11]. In contrast, animal models reveal preventative treatment diminishes tumor development [11]. Confirming the importance of MMP1 in a subset of OCs could improve survival by identifying patient populations that will respond best to treatment strategies targeting MMPs.
It is important to remember that in vivo, stromal cells, epithelial cells, and endothelial cells all contribute to the MMPs found in the extracellular environment [48]. This concept doesn’t diminish the importance of altered MMP expression by BRAT cells. Instead, the potential exists for even greater enhancement of motility and invasive capability. MMP1 can be activated by multiple other MMPs that may be present in the ECM from any cell source. Further, MMP1 activates several other proteases that cleave substrates it cannot. For example, MMP1 aids in activation of MMP2, which cleaves collagen IV, a major constituent of the ovarian basement membrane. Regardless of the mechanism, alteration of MMP activity likely has far-reaching consequences for the local ECM and epithelial-stromal interplay in vivo.

In these cells, BRAT-mediated changes conducive to development of a malignant phenotype may have begun. It is interesting to speculate that BRAT cells, though non-tumorigenic in their current state [18], may represent a step forward on the continuum of cellular malignancy. Loss of DNA damage repair through BRCA1 mutation and LOH as well as gain of function mutant activities such as gene regulation both likely contribute to further accumulation of genetic changes that promote OC progression and are characteristic of late stage BRCA1 mutation-associated OC.

I have identified another downstream target of the 185delAG BRCA1 mutant, BRAT. MMP1 expression is increased in BRAT cells transcriptionally in a c-Jun-dependent mechanism, and BRAT cells exhibit increased MMP1 secretion. Mutation-associated changes early in OC development could poise cells in normal tissue of mutation carriers for transformation or acquisition of invasive or metastatic ability.
Further exploration of these changes can increase our understanding of early steps of OC development and help identify potential screening and treatment strategies.
References


Chapter 3:

Impact of BRAT on Apoptosis, Gene Regulation, and Migration in Normal Breast Epithelial and Breast Cancer Cells

Introduction

Though BRCA1 mutation carriers have a slightly elevated risk for cervical, uterine, and pancreatic cancer [1], the most significant cancer risk associated with a BRCA1 mutation is for breast and OC [2]. Though it is not known why the disease manifests preferentially in these tissues, several hypotheses have been put forth: 1) BRCA1 mutation results in impaired DNA damage repair, accumulation of DNA damage, and apoptosis. Cells in the breast and ovary, however, could have a mechanism by which to delay apoptosis and accumulate mutations, resulting in cell survival and tumorigenesis [3]; 2) Higher rates of LOH may occur in the breast and ovary [3], which could result in loss of wt function and emphasize the importance of mutant BRCA1 functions in these tissues; and 3) Increased risk for breast and OC may result in part from the influences of hormone signaling. Estrogen is clearly important for normal breast development and lactation and also in sporadic breast cancer. Expression of estrogen receptor alpha, which mediates the proliferative effects of estrogen for breast epithelial cells, is over-expressed in more than half of sporadic breast cancers [4], and selective estrogen receptor modulators, such as tamoxifen, are highly effective in treating breast cancers that express estrogen receptor alpha. Despite the fact that most BRCA1-
associated breast tumors are estrogen receptor alpha negative [5], evidence indicates an interplay between BRCA1 and estrogen signaling. BRCA1 inhibits estrogen receptor alpha’s ligand-dependent transactivation function and down-regulates the co-activator p300 (E1A binding protein 300) (Reviewed in [6]). Consequently, aberrant estrogen signaling in the breast and ovary, either directly or indirectly through paracrine signaling, may contribute to cancer in these tissues by enhancing survival despite impaired DNA damage repair due to BRCA1 mutation (Reviewed in [2]).

Very few epidemiologic studies have attempted to determine whether specific BRCA1 mutations confer differential risk for breast or OC. Individuals who carry the same BRCA1 mutation may develop either breast or OC, while some individuals develop both. In population studies, families with predisposition to both breast and OC exhibit variation in the ratio of breast to OC occurrence [7], possibly as a result of the mutation they share. These observations suggest specific mutations have tissue-specific functions that contribute to risk of cancer in the breast and/or ovary. Neuhausen et al. did not observe a significant difference in proportion of OC and breast cancer incidence mediated by specific mutations [8]. In contrast, utilizing a population of 191 Ashkenazi Jewish OC patients, Moslehi and colleagues compared the estimated risk for breast or OC to 75 years of age mediated by three founder mutations. The three mutations conferred a similar risk of breast cancer, however risk of OC was greater in 185delAG mutation carriers compared to 5382InsC and 6174delT carriers (odds ratio 36.6, 20.8, and 14.2 respectively) [9]. Differential risk of OC mediated by specific mutations may result from gain of function activity mediated by BRCA1 mutants in the ovary, while similar functions are not conferred, or have less impact in the breast.
Studies estimating risk levels for specific mutations are inherently difficult to interpret, however, because risk estimates are dependent on the study population. For example, risk estimations are higher in studies involving high risk families, and depend on biologic factors such as prophylactic oophorectomy or mastectomy and age [10]. Environmental factors such as diet or oral contraceptive use further complicate risk assessment. As a result, estimated ranges of risk associated with BRCA1 mutation for ovarian or breast cancer are wide, and statistical differences in risk between specific mutations are not typically reported [10].

Larger studies examining mutations grouped by their location within the BRCA1 gene have attempted to strengthen epidemiologic analysis. Such studies have reported differential ovarian and breast cancer risk based on the location of truncation mutations within the BRCA1 gene. For example, truncation and splice altering mutations in the 5’ third of the gene (before nucleotide 2401) and the 3’ third of the gene (after nucleotide 4191) contribute to a significantly higher proportion of breast to OC incidence [7, 11]. Conversely, Thompson et al. identified a region between nucleotides 2401 and 4190 in which mutations contribute to a higher proportion of OC to breast cancer [12]. The location of a truncation mutation within the gene determines the predicted truncated protein size, which potentially influences the function of the mutant within the breast or ovary and, for some mutants, the relative ratio of breast to OC associated with a specific mutation. Indeed, clinical disease characteristics have been shown previously to be influenced by mutation location within the gene. For example, mutations in the 5’ and 3’ portions of the adenomatous polyposis coli (APC) gene result in a less severe version of familial adenomatous polyposis [11].
I have previously observed that the BRAT mutation confers molecular and cellular changes in HOSE that may promote OC development. In order to understand the role of BRAT function in tissue specific disease and to determine whether BRAT’s unique functions may preferentially promote OC, I examined the cellular and molecular impact of BRAT in normal breast epithelial and breast cancer cells. I have investigated several cellular and molecular processes previously found to be influenced by BRAT in normal HOSE or OC cells, including apoptosis and gene regulation, as well as cell migration.

Methods

**Cell culture and transfection.** MCF7, SkBr3, and MDA-MB-231 human breast cancer cells were cultured in Medium 199/ MCDB 105 (Sigma, St. Louis, MO) with 10% fetal bovine serum and gentamicin. MCF10A normal human breast epithelial cells were cultured in DMEM/F12 (Mediatech, Manassas, VA) supplemented with 15mM HEPES, insulin 10ug/mL, EGF 20ng/mL (Sigma, St. Louis, MO), cholera toxin 100ng/mL (Biomol, Plymouth Meeting, PA), hydrocortisone 0.5ug/mL (BD Biosciences, Sparks, MD), L-glutamine (MP Biomedicals, Solon, OH), glucose, sodium bicarbonate, 10% fetal bovine serum and gentamicin. All cells were incubated at 37°C with 5% CO₂. Two million cells were transiently transfected as previously described [13] using Program X-005 (for MCF7 cells), E-09 (for SkBr3 cells), X-013 (for 231 cells), T-024 (for MCF10A cells), Kit V, and the Nucleofector device (Amaza/ Lonza, Walkersville, MD) with 2.5-3.5 ug of plasmid (GFP, PCDNA3.1 or Flag-BRAT [13]).
**Cell viability assay.** Twenty-four hours after transfection, cells were trypsinized, counted, and plated at sub-confluence in triplicate in 96 well plates with media containing vehicle or 2-10uM cisplatin. After 1 hour, cells were assayed using the CellTiter 96® AQueous One Solution Cell Proliferation MTS (Promega, Madison, WI) colorimetric assay according to manufacturer’s instructions. Absorbance was read using an ELx800 microplate reader (Bio-Tek Instruments, Winooski, VT) and designated “0h.” Cells were then assayed every 24 hours for 72 hours. Results were expressed as the mean absorbance ± standard error.

**Western blot.** Cells were PBS washed, trypsinized, pelleted, and washed 1-2 times in cold PBS. Cells were lysed for 30 minutes on ice in modified CHAPS buffer, and lysate was centrifuged at 115,000 xg, at 4° C, for 1h. Thirty to 100μg of protein were separated via 10% SDS-PAGE. Proteins were transferred to PVDF membranes, dried at 37° C for 1 hour, and blocked in 5% milk or bovine serum albumin in Tween 20-TBS. Blots were incubated in their respective antibodies overnight, followed by incubation with an HRP-conjugated secondary (Fisher Scientific), and developed via ECL (Pierce). Antibodies: BRCA1 (Calbiochem), cleaved caspase 3, caspase 3 (Cell Signaling Technology, Beverly, MA), actin (clone AC-40, Sigma, St. Louis, MO), and maspin (BD Biosciences, San Jose, CA).

**RT-PCR.** RNA samples were isolated using TRIzol reagent from Invitrogen (Carlsbad, CA) per manufacturer’s protocol and DNAsase treated. For semi-quantitative PCR, one microgram total RNA, oligo(dT), and reverse transcriptase were used to generate single-strand cDNA as previously described [13]. The cDNA samples were amplified using the Applied Biosystems GeneAmp RNA PCR Core Kit (Foster City,
CA). Primers used were: Flag-BRAT sense (CGATGACAAAATGGATTTATCTGC), Flag-BRAT antisense (GAGACAGGGTCTCCATCAACTCC), MMP1 sense (GAGCAAAACATCTGAGGTACAGGA), MMP1 antisense (TTGTCGGATGATCTCCCTGACA) [14], actin (98 base pairs) sense (GGGAATTCAAAACTGGAACGGTGAAGG), and actin (98 base pairs) antisense (GGAAGCTTATCAAAGTCCCTCGGCCACA) Maspin sense (GGAGGCCACGTTCCTGTAT) and Maspin antisense (CCTGGCACCTCTTATGGA). The amplified products were separated by electrophoresis on a 10% polyacrylamide gel, stained with SYBR Green (Lonza, Rockland, ME), and photographed with the Kodak EDS 120 Digital Analysis System. The net intensity of each band was normalized to the respective endogenous control band. For real time PCR, one hundred ng total RNA was reverse transcribed to generate single-strand cDNA as previously described [13]. The cDNA samples were amplified using Fast SYBR Green Master Mix (Applied Biosystems) on an Applied Biosystems Step One Plus instrument. RQ (relative mean mRNA expression level) was calculated by the Step One software version 2.0. Using standard curves constructed for target and endogenous control genes, an arbitrary quantitative gene expression value was determined from the Ct for each gene from each sample. Target gene values were normalized to control gene values, and fold difference was determined by dividing by the designated reference/ calibrator sample.

**Scrape Assay.** Following transfection, cells were plated at near confluence in 60 millimeter dishes. Sixteen to twenty-four hours later, cells were washed one time with PBS, and a scrape was made down the center of the dish using a sterile rubber cell
scraper. Cells were washed 2 times with PBS, and imaged immediately and every 24 hours for 72 hours using an Olympus 1X71 microscope with D870 camera.

Statistics. Samples for MTS assays were run in triplicate, and the data were subjected to the Student’s t test for determination of statistical significance.

Results

Endogenous BRCA1 and exogenous BRAT expression levels in normal breast epithelial and breast cancer cells. Etiologic studies have revealed a possible role for hormones and their receptors in BRCA1-associated breast cancer. For example, earlier age at first pregnancy, parity, and later first menarche decrease breast cancer risk in non-mutation carriers, however, studies have shown opposite or absent trends in BRCA1 mutation carriers [6]. In contrast, breast feeding reduces risk and oral contraceptive use increases risk similarly to non-mutation carriers [6]. Prophylactic oophorectomy, which eliminates a major source of estrogen production before menopause, significantly reduces breast cancer risk in mutation carriers as well [15].

For this reason, multiple breast cancer cell lines, estrogen receptor positive and negative, were chosen for analysis. Each cell line represents human breast adenocarcinoma cells isolated from pleural effusion fluid, including: MCF7 cells are estrogen receptor positive, tumorigenic and metastatic in nude mice [16-18]. SkBr3 cells are tumorigenic, estrogen receptor negative, and overexpress Her2[19] [20]. MDA-MB-231 cells are tumorigenic and estrogen receptor negative [16], and used as a model for highly aggressive breast cancer. One normal breast epithelial cell line, MCF10A was also analyzed. MCF10A cells are a spontaneously immortalized sub-population of normal,
non-tumorigenic breast epithelial cells derived from a patient with fibrocystic disease [16] that are frequently utilized for comparison with breast cancer cell lines.

The breast cancer cell lines utilized exhibit varying levels of BRCA1 expression. MCF7 cells are reported to have genomic loss of one BRCA1 allele [21] and to express low levels of BRCA1 mRNA and protein [22]. SkBr3 cells are also reported to express low basal levels of BRCA1 [23]. The studies reporting BRCA1 levels in these cell lines have utilized different methods of detection (i.e. RT-PCR for mRNA level, Western blotting for protein level), and different reagents. To determine the relative expression of BRCA1 in the cell lines utilized, lysates were collected from each cell line and Western blotting was performed. SkBr3 cells expressed the highest levels of BRCA1, followed by MCF7 cells, MDA-MB-231 cells, and MCF10A cells (Figure 3.1).

Optimal transfection conditions for cell number, amount of DNA, and electroporation parameters were elucidated by transfection of GFP and followed by microscopic or flow cytometric analysis of the percentage of transfected cells (transfection efficiency). Transfection efficiencies were approximately 40-70% (Figure 3.2.). Transfection of BRAT was then performed under the optimized conditions for each cell line and confirmed by semi-quantitative PCR. Figure 3.3. illustrates a representative figure confirming the 120 nucleotide BRAT PCR product efficiently expressed in SkBr3 cells from 24-72 hours after transfection. Expression was confirmed in each remaining cell line (data not shown).

**BRAT does not significantly impact proliferation or chemosensitivity of normal breast or breast cancer cells.** Previous studies from our lab have shown that expression of BRAT in normal HOSE and OC cells increases the sensitivity of these cells
Figure 3.1. Wild type BRCA1 levels in normal human breast epithelial and breast cancer cells.
Cells were plated at equal densities, collected, and lysed. Protein lysate was separated using SDS-PAGE and Western blotting was performed using the indicated antibodies. Relative band intensity was determined by dividing the BRCA1 band intensity by the actin band intensity for each lane.
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<th>Cell Line</th>
<th>Method</th>
<th>Estimated efficiency</th>
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<tr>
<td>SkBr3</td>
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<td>60%</td>
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<tr>
<td>MDA-MB-231</td>
<td>Microscopy</td>
<td>50%</td>
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<tr>
<td>MCF10A</td>
<td>Microscopy</td>
<td>70%</td>
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<tr>
<td>MCF7</td>
<td>Flow cytometry</td>
<td>42%</td>
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**Figure 3.2.** Transfection efficiencies of normal breast epithelial and breast cancer cell lines.

Indicated cell lines were transfected with GFP using the appropriate Amaxa nucleofector program. After 24 hours, cells were imaged using fluorescent microscopy, and percent GFP positive cells was estimated. Representative fluorescence images at 10X magnification for each cell line are pictured with the concomitant phase contrast image. For flow cytometry, cells were collected by centrifugation and resuspended in PBS. Living cells were gated for GFP positivity.
Figure 3.3. BRAT is efficiently expressed in SkBr3 cells. SkBr3 cells were transiently transfected with BRAT plasmid, and cells were collected 24-72 hours after transfection. RNA was isolated, DNase treated, reverse transcribed, and semi-quantitative PCR was performed for BRAT. PCR products were electrophoresed on a 10% acrylimide gel, stained with SYBR green, and imaged.
to cytotoxic drug treatment [24]. Therefore, to test whether BRAT impacts chemosensitivity of breast cancer cells and normal breast epithelial cells, the MTS assay was performed on vehicle and cisplatin treated cells. No significant change in cell viability was observed in MCF7 cells treated with vehicle or 10uM cisplatin up to 72 hours after transfection (Figure 3.4. A). Similar results were observed in MDA-MB-231 and MCF10A cells (data not shown). SkBr3 cells transfected with BRAT exhibited cell viability similar to control PCDNA transfected cells (Figure 3.4. B). However, cisplatin-mediated cytotoxicity was 17% greater in SKBr3 BRAT cells at 24 hours. The decrease in BRAT cell viability was most pronounced at 48 hours (24%), and a moderate decrease was still evident at 72h (15%) (Figure 3.4. B).

The enhanced chemosensitivity observed in BRAT HOSE cells was previously found to involve induction of apoptosis through caspase 3 activation [13, 24]. Therefore, to further investigate the effect of BRAT on chemosensitivity of SkBr3 cells and determine whether caspase 3 is involved, Western blotting was performed to measure the level of cleaved (activated) caspase 3. No significant difference was observed in cleaved caspase 3 levels of BRAT-transfected SkBr3 cells compared to control PCDNA SkBr3 cells when treated with vehicle or cisplatin (Figure 3.4. C). This data suggests that BRAT does not enhance apoptosis by caspase 3 activation in SkBr3 breast cancer cells.

The transfection efficiency of 118 HOSE was previously found to be greater than 97% [24]. Transfection of MCF10A and breast cancer cells was efficient, but not as high (Figure 3.2.). To rule out the possibility that BRAT failed to modulate chemosensitivity because of lower transfection efficiency and, therefore, copy number in transiently transfected cells, stable cell lines were generated. SkBr3 cells were transfected
Figure 3.4. BRAT does not significantly impact growth or chemosensitivity of breast cancer cells.

A. MCF7 or B. SkBr3 cells were transiently transfected with PCDNA or BRAT and plated in 96 well plates. Proliferation was measured via MTS assay at indicated time points on triplicate samples of untreated or 10 µM cisplatin-treated cells. Graphs illustrate average absorption at 450 nm ± SE. 

C. SkBr3 cells were transiently transfected with PCDNA or BRAT and plated. Cells were treated with 10 uM cisplatin for the indicated time point, lysed, and protein lysate was electrophoresed on a 10% SDS-PAGE gel. After transfer to PVDF membrane, blotting was performed with indicated antibodies. Relative band intensity was calculated by dividing cleaved caspase 3 band intensity by actin band intensity for each lane.
with PCDNA or BRAT and maintained in G418 selection media. BRAT expression was confirmed by semi-quantitative PCR to be similar to or higher than that of 118 stable cells (Data not shown). SkBr3 cells stably expressing BRAT exhibited 14% decrease in viability compared to PCDNA3.1 cells after 48 hours of cisplatin treatment, though viabilities were similar to PCDNA cells at other time points (data not shown). This data suggests that the BRAT mutation mediates ovary-specific effects on cell growth and chemosensitivity.

**BRAT does not significantly impact maspin expression in normal breast epithelial or breast cancer cells.** In addition to its impacts on chemosensitivity in HOSE cells, BRAT was previously found to increase expression of the 42 kDa serine protease inhibitor, maspin. Maspin is unable to undergo the conformational change necessary for normal serpin function [25]. Maspin was identified by its diminished expression in breast tumor samples compared to normal breast tissue [26]. Interestingly, maspin expression is low in normal HOSE, but is uniquely up-regulated in OC and correlates with high tumor grade and shorter overall survival [27, 28].

In agreement with maspin’s aforementioned clinical correlations in breast cancer, maspin has the potential to impact several important processes in breast cancer cells. Increased maspin is associated with enhanced drug sensitivity in breast cancer models [29]. Maspin has been shown to increase adhesion and diminish invasion and metastasis [30, 31] of breast cancer cells as well. Therefore, I next sought to determine whether BRAT impacts maspin expression in normal human breast epithelial cells and breast cancer cells as it does in HOSE cells.
To this end, I first performed Western blotting to determine basal levels of maspin in each cell line. As expected, maspin expression was robust in normal MCF10A cells and undetectable in MCF7, SkBr3, and MDA-MB-231 breast cancer cells (Figure 3.5. A). Transfection of BRAT into MCF10A cells did not significantly alter maspin mRNA levels as measured by semi-quantitative PCR (Figure 3.5. B). Further, transfection of BRAT into SkBr3 cells did not result in detectable maspin expression (Figure 3.5. C), suggesting that BRAT does not significantly modulate maspin expression in normal human breast epithelial cells, nor does BRAT activate maspin expression in human breast cancer cells.

**BRAT does not significantly alter MMP1 expression levels in normal breast epithelial or human breast cancer cells.** My data indicate elevation of the gene encoding matrix metalloproteinase 1 (MMP1) in HOSE cells transfected with BRAT (Chapter 2). MMP1 is clearly important for breast cancer progression. MMP1 gene expression is elevated in breast tumor tissue from tumors that metastasized to bone compared to tissue from non-metastatic breast cancer [32]. Further, inhibition of MMP1 enhances apoptosis and inhibits metastasis of human breast cancer cells from the mammary fat pat of nude mice [33]. Because BRAT regulates MMP1 expression in normal HOSE cells, and because of a possible role for MMP1 in breast cancer progression, I evaluated the impact of BRAT on MMP1 expression in normal breast epithelial and human breast cancer cells.

MMP1 expression was undetectable in MCF10A, SkBr3, and MCF7 cells by real time PCR, and transfection of BRAT did not result in increased expression (data not shown). Similarly, MMP1 expression was not detected by semi-quantitative PCR in
Figure 3.5. BRAT does not significantly impact maspin expression in breast cancer cells. A. Cells were plated at equal densities, collected, and lysed. Protein lysate was separated using SDS-PAGE and Western blotting was performed for maspin. MCF10A (B.) or SkBr3 (C.) cells were transiently transfected with BRAT plasmid. Cells were collected 24-72 hours after transfection. RNA was isolated, DNase treated, reverse transcribed,
and semi-quantitative PCR was performed for maspin and actin. PCR products were electrophoresed on a 10% acrylimide gel, stained with SYBR green, and imaged.
MCF10A or SkBr3 cells (Figure 3.6.). While a 185 nt PCR product representing MMP1 mRNA was detectable in MDA-MB-231 cells, levels were not significantly altered by expression of BRAT (Figure 3.6.), suggesting MMP1 up-regulation is a tissue-specific function of the BRAT mutation.

**BRAT does not significantly impact migration of breast cancer cells.** Though BRAT did not appear to significantly impact apoptosis or regulation of targets previously identified in HOSE, it has not yet been determined whether BRAT impacts migration in any cell system. MMP1 is a major contributor to extracellular matrix remodeling, cell migration, invasion, and metastasis, therefore, in addition to MMP1 regulation, I chose to investigate whether BRAT modulates cell migration of breast cancer cells. To this end, a scrape assay was performed. For MDA-MB-231 and MCF7 cells, images of scraped edge appeared similar for PCDNA and BRAT-transfected cells (data not shown). SkBr3 control PCDNA cells exhibited slightly more frequent patches of cells growing within the scraped area compared to BRAT cells (Figure 3.7.), however, changes were minimal.

These data suggest BRAT does not significantly alter breast cancer cell migration.

**Discussion**

BRCA1 mutation-associated breast cancers are significantly more aggressive than their sporadic counterparts. Mutation-associated tumors are frequently ductal carcinomas [5], highly proliferative [34], of a higher grade [35, 36], exhibit enhanced genomic instability [34], and result in poorer survival than sporadic breast cancer [37]. Like BRCA1 mutation-associated OC, BRCA1-associated breast cancer cells are more sensitive to cisplatin treatment [37]. BRCA1-associated tumors are also more frequently estrogen receptor alpha, progesterone receptor, and Her2 negative [5], characteristics that
**Figure 3.6.** MMP1 expression is not altered by BRAT in normal breast epithelial or breast cancer cells. Indicated cells were transiently transfected with BRAT plasmid. Cells were collected 24 hours after transfection. RNA was isolated, DNase treated, reverse transcribed, and semi-quantitative PCR was performed for MMP1 and actin. PCR products were electrophoresed on a 10% acrylamide gel, stained with SYBR green, and imaged.

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**Figure 3.7.** Migration of SkBr3 cells is not significantly impacted by BRAT. SkBr3 cells were plated at near confluence, allowed to attach overnight, and a single continuous scrape was made down the center of the dish. Detached cells were removed and remaining adherent cells were washed with PBS and imaged at 4X immediately and every 24 hours for 72 hours.
are frequently associated with more aggressive disease and preclude treatment with anti-estrogens. In contrast to BRCA1-associated OCs, BRCA1-associated breast cancers exhibit a unique gene expression profile, including up-regulation of DNA repair genes, such as Rad51 and human mutS homolog 2 (MSH2) [38], and genes associated with a basal-like tumor histology, such as cytokeratin 5 and 17 (Reviewed in [39]).

Because of the aggressive clinical nature of BRCA1 mutation-associated breast cancers, decisions about screening and prophylaxis for mutation carriers are critical. Therefore, it is important to understand clinical characteristics specific to each mutation. For example, classification of mutations as having low penetrance for breast cancer would afford carriers the choice to avoid radical prophylactic procedures, such as mastectomy. Instead of grouping all BRCA1 mutations, clinical data such as tumor histology, breast/ovarian specificity, and aggressiveness should be analyzed for each mutation. Further, the molecular changes that mediate tumor initiation and progression are likely dependent on BRCA1 mutant function, and are, therefore, specific to each mutation.

In addition to epidemiologic data for each BRCA1 mutation, study of mutant functions in cell and animal model systems will reveal mechanisms by which each mutation promotes cancer in the breast or ovary and aid in identification of targets for more effective treatment of mutation-associated breast and OC. To this end, specific BRCA1 mutants have been shown previously to impact important cellular processes in breast epithelial and breast cancer models through gain of function or dominant negative activities. For example, mouse mammary gland-specific expression of wt BRCA1 delays mutagen-induced tumors, however, expression of a BRCA1 splice variant lacking the
first 72 amino acids accelerates tumorigenesis and death of transgenic mice [40]. A synthetic BRCA1 truncation mutant encoding the first 300 BRCA1 amino acids inhibits mammary gland differentiation and development in wt mice [41]. HCC1937 breast cancer cells are homozygous for the 5382InsC mutation, carry a p53 mutation with concomitant loss of the wt allele, and exhibit deletion of PTEN and other genetic aberrations implicated in breast cancer [42]. When transfected with wt BRCA1, these cells exhibit chemo-resistance, however, resistance is reversed upon co-transfection of C terminal BRCA1 mutants [43]. Co-expression of 5382InsC and 5677InsA with wt BRCA1 in breast cancer cells exerts a dominant negative effect by inhibiting the wt protein’s ability to enhance apoptosis [44].

Of the three breast cancer cell lines tested, only the SkBr3 breast cancer cell line exhibited an increase in cisplatin sensitivity. Interestingly, this cell line over-expresses Her2, a member of the EGF receptor family that has no specific ligand, but preferentially dimerizes with and activates other family members. This dimerization amplifies activation of MAPK and PI3K pathways and promotes survival, proliferation, migration, invasion, metastasis, and angiogenesis [45]. Her 2 is over-expressed in 25-30% of all breast and OCs [46], and correlates with poor prognosis [47]. As mentioned previously, Her2 is frequently absent in BRCA1 mutation-associated breast cancers, however, data is unavailable for each mutation. It would be interesting to examine whether Her2 over-expression is associated with some BRCA1 mutations and not others in tumor samples as well as whether Her2 promotes or cooperates with mutant BRCA1 functions in mutation-associated breast and ovarian model systems.
In contrast, some BRCA1 mutants do not mediate significant activity in breast cancer models independent of the loss of wt BRCA1 function. HCC1937 cells are more sensitive to ionizing radiation compared to breast cancer cells without a known BRCA1 mutation, but restoration of wt BRCA1 partially reverses this sensitivity. Interestingly, expression of other BRCA1 mutants, M1775R, T826K, MfeΔ (frameshift at codon 1604), C64G, and P1749R, does not reverse sensitivity [48]. While wt BRCA1 reduces hyper-recombination and enhances homologous DNA repair in MCF7 cells, which have a single wt BRCA1 copy, the 5382InsC mutation does not mediate these functions [49]. A breast epithelial cell line has been established that harbors one wt BRCA1 copy and one copy of the 185delAG mutation [50]. Analysis of this line revealed no detectable difference in growth, anchorage independence, response to ionizing radiation and hydrogen peroxide treatment, or tumorigenicity compared to breast epithelial cells with two wt copies of BRCA1 [50].

Though most studies investigate mutant function in only one model system, Holt and colleagues compared the effect of two BRCA1 truncation mutants of 340 and 1835 amino acids on growth of breast and OC cells. In agreement with my findings, cell type specific effects were observed. The mutants did not significantly impact growth of breast cancer cells, however, growth inhibition was observed in three OC cell lines [21]. You et al. found cell type-specific BRCA1 mutant functions, as well. While a synthetic BRCA1 mutant lacking the N-terminal 302 amino acids enhanced apoptosis and inhibited growth of MCF10A cells, growth of HeLa cells was unaffected [51].

Reproductive organs and breast tissues are sometimes grouped together under a broad categorization of “hormone-responsive” tissue, and the intricate differences in
these tissues are overlooked. Tissue-specific effects in the context of cancer are a concept that has been demonstrated before. For example, tamoxifen is categorized as a selective estrogen receptor modulator because it exerts growth inhibitory effects on breast cancer cells and stimulatory effects on the uterine lining [52]. These disparate impacts are likely related to the complement of transcription factors and co-regulators expressed in each tissue type. Likewise, similar differences likely dictate the function of BRCA1 mutant proteins by providing different expression and stoichiometry of interacting proteins as well as redundancy for pathways inhibited by mutant function.

Changes in gene regulation are observed in BRCA1 mutation carriers, and likely contribute to tissue-specific mutant function as well. Indeed, comparative genomic hybridization revealed that amplifications or deletions of specific chromosome regions was significantly more frequent in breast tumors from mutation carriers versus control tumors [53]. Conversely, Jazaeri et al. found similar patterns of gene expression in BRCA1/2 mutant ovarian tumors compared to sporadic tumors [54]. Whether these changes in expression are correlative or occur as a result of BRCA1 mutation, there is great potential for differential mutant BRCA1 protein function as a result of differential tissue-specific gene expression.

Lastly, splice variants of the BRCA1 gene have been confirmed [55]. It has been suggested that tissue-specific expression of 3’ splice variants could result in restoration of wt protein expression [7]. Tissue-specific alteration of the balance of mutant and wt protein or repression of the mutant protein in this manner could result in significant impacts on cell physiology. Indeed, BRCA1 mutations in the 3’ third of the gene result in a significantly lower proportion of OC compared to breast cancer incidence [7].
However, it has been difficult to determine differences in risk associated with specific mutations because of limited sample numbers.

It is clear from this study, then, that the BRAT mutation confers tissue-specific effects on apoptosis and gene regulation. Tissue specific mutant functions may contribute significantly to differential breast and OC risk and penetrance. It remains to be determined whether other BRCA1 mutations confer similar functions and, therefore, similarly impact cancer risk in the breast and ovary. Elucidating the mechanism by which mutations confer enhanced cancer risk in the breast and ovary will allow for a better understanding of mutation-associated cancer etiology and therefore better treatment for mutation carriers. Further, this information will improve prognostic accuracy and allow physicians and patients to make better decisions regarding treatment and prophylaxis.

We have previously reported BRAT-mediated changes in normal HOSE and OC cells that modulate cytotoxicity and regulation of genes potentially important in BRAT-associated OC. Here, I compared BRAT’s impact on these cellular processes in normal human breast epithelial and breast cancer cells. I found that BRAT’s impacts on apoptosis and regulation of MMP1 and maspin are specific to the ovary, and the BRAT mutation likely increases risk of cancer in the breast by an alternative and yet undiscovered mechanism.
References


Chapter 4:
Conclusions

Origins of BRCA1-associated Ovarian Cancer

A better understanding is needed of the mechanism by which normal HOSE of BRCA1 mutation carriers becomes malignant. Models of several cancer types, such as colon cancer, employ the concept of early “pre-malignant” molecular and histologic changes that promote tumorigenesis. Because OC is frequently diagnosed at late stage, identification and characterization of a pre-malignant state has proven difficult, however, studies suggest histologic and cytologic changes in non-tumor ovarian tissue of patients with a family history of OC or a confirmed BRCA1 mutation. For example, more frequent deep invaginations in the ovary surface, dysplasia, hyperplasia, and/ or surface papillae have been observed in prophylactically removed ovaries versus normal ovaries [1-3]. These abnormal regions are hypothesized to be the origin of epithelial OC.

The role of BRCA1 mutations in the development of histologically abnormal regions is not yet understood, though the high frequency of these regions in mutation carriers suggests a role for BRCA1 mutants. Indeed, carcinoma was found to originate in inclusion cysts of several prophylactically removed ovaries of mutation carriers, and the same p53 mutation was found in tumor tissue and tumor-adjacent dysplastic and normal surface epithelium [4]. This evidence supports the hypothesis of morphologically abnormal regions of HOSE as BRCA1-associated OC precursors, and for p53 mutation
and BRCA1 LOH as early events in BRA1 mutation-associated OC development [4]. Similar findings of a p53 “signature” have been reported in prophylactically removed fallopian tube epithelium from BRCA1 mutation carriers, though this phenomenon was detected in a similar proportion of BRCA1 mutation carriers and control patients [5-7].

HOSE cells stably expressing BRAT have previously been characterized as non-tumorigenic by lack of growth in soft agar and lack of telomerase activity [8]. Though BRAT cells do not exhibit malignant characteristics, it is possible that changes conducive to development of a malignant phenotype have begun. I propose that BRAT cells, like the ovarian surface epithelium of BRCA1 mutation carriers, may represent forward movement on the continuum of cellular malignancy. Additional changes or mutations may be necessary for cells to become malignant. Indeed, the model I propose does not exclude the contribution of other oncogenes, tumor suppressors, or invasion/metastasis-promoting proteins. Loss of DNA damage repair through BRCA1 mutation and LOH as well as gain of function mutant activities such as gene regulation both likely contribute to further accumulation of genetic changes that promote OC progression and are characteristic of late stage BRCA1 mutation-associated OC.

Future Studies

The role of BRAT and other BRCA1 mutants in early pre-malignant changes conducive to OC development requires further investigation. First, it is crucial to identify other BRCA1 mutants that mediate gain of function or dominant negative activity and the specific pathways and contexts in which they function. Other BRCA1 mutations found to elude nonsense-mediated RNA decay, such as 5382insC, 5677insA, 188del11, and Arg1835ter [9], are promising avenues for further study. Interestingly, the 188del11
mutation results in a STOP codon at codon 39 and a predicted protein product of similar size to BRAT, and may, therefore, mediate similar functions and specificity for ovarian cells. The stability of BRCA1 mutant mRNAs should also be tested in breast and ovarian cell lines, because previous studies were performed in lymphoblastoid cells [9]. Similarly, it is critical to continue seeking BRCA1 mutant proteins in cell lines and clinical specimens, as some mutant proteins may exhibit instability [10].

As demonstrated by our studies, tissue specificity is an important factor in BRAT function. It is likely that other mutants besides BRAT mediate tissue-specific effects. HOSE-118 cells would be an ideal model in which to determine the impact of other BRCA1 mutations that exhibited gain of function activity in other model systems. For example, 5083del19, which increased periostin expression in HeLa cells and breast cancer tissue [11], may regulate other genes in the ovary.

Cellular processes previously demonstrated to be altered by BRCA1 mutants provide direction for identifying additional downstream targets. For example, several of the genes found by microarray to be differentially regulated in BRAT-expressing HOSE cells encode proteins that localize to the extracellular space and are potentially important in OC motility, invasion, and metastasis (collagen I, and collagen III, IL-6, IL-1alpha, IL-1beta, and MMP1). IL-1α and β expression is increased, and the pro form of IL1-β is also found at higher levels in conditioned media of BRAT cells compared to PCDNA cells (Figure 4.1). As mentioned previously, IL-1 enhances MMP1 mRNA expression and stability [12] and promotes HOSE cell proliferation indirectly by up-regulating the mitogenic factor TNFα [13]. Further, as demonstrated by our studies, BRCA1 mutation carrier-derived normal cell lines can reveal mutant functions that promote early
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<td>Si Con</td>
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**Pro-IL 1β**

**Amido Black Stain**

**Figure 4.1.** Pro-IL1β levels are increased in conditioned media of BRAT cells. HOSE 118 cells were transiently transfected with indicated transfectant. Cells were serum starved in media with 0.1% FBS for 24 hours. Conditioned media was collected, concentrated 47-fold, and run on a 10% SDS-PAGE gel. Membrane was blotted with IL1β antibody, and subsequently stained with Amido Black for total protein.
pre-malignant changes and transformation (Chapter 2), while expression of BRCA1 mutations in cancer cell models can reveal mutant functions that impact cancer progression through apoptosis [14], growth, invasion, or metastasis.

BRAT’s effects on apoptosis and MMP1 gene regulation are likely linked as well, as up-regulation of both maspin and MMP1 involves c-Jun. In addition to Akt’s importance in enhanced chemosensitivity of BRAT cells, preliminary data also indicate a role for Akt in MMP1 up-regulation. Future studies will continue to explore Akt’s involvement in BRAT-mediated MMP1 up-regulation, and determine whether the MAPK signaling cascade is also affected by BRAT. It remains to be determined whether these and other as-yet undiscovered functions of BRAT will share common mechanisms or signaling pathways.

Cell models allow mechanistic studies of BRCA1 mutant function at the molecular level, however, discerning the physiologic impact of BRCA1 mutant functions in vivo is vital to establishing clinical relevance. Staining of BRAT mutation-associated OC tissue and adjacent normal HOSE from patients is currently ongoing to determine whether MMP1 expression is elevated in these tissues compared to tissue from patients without a family history of OC. Though sample size will be limited, this data will confirm the clinical relevance of BRAT-mediated MMP up-regulation.

To execute additional in vivo studies, a transgenic mouse model heterozygous for BRAT or other risk-associated BRCA1 mutations could be created to confirm the presence of pre-malignant changes similar to those found in human mutation carriers. BRCA1 heterozygous knockout mice do not spontaneously develop mammary or ovarian tumors [15], possibly because the mouse lifespan is not long enough to accumulate the
additional genetic changes necessary to achieve tumorigenesis [15]. BRCA1 knockout mouse models also fail to recapitulate BRCA1 mutant protein functions that likely contribute to tumorigenesis. In contrast, a transgenic BRCA1 mutant mouse model would address this shortcoming. Indeed, BRCA1 variants besides wt contribute significantly to mammary tumor development, as BRCA1 knockout mice that retain the Δexon11 develop tumors that are morphologically and genotypically distinct from knockout models designed to eliminate the entire BRCA1 gene [15].

BRCA1 mutation-associated breast and OCs exhibit frequent chromosomal aberrations and it is clear that accumulation of additional mutations is necessary for tumorigenesis. For example, mouse mammary gland-specific BRCA1 knockout mice that harbor a p53 mutation develop tumors with shorter latency [16]. It would be informative to investigate latency and tumor characteristics of BRCA1 mutant transgenic mice with concomitant overexpression of oncogenes previously shown to be important in OC, such as Ras, epidermal growth factor receptor (EGFR), or Her2, or in the context of a p53 mutant background. Transgene expression specific to the reproductive epithelium has been achieved using the Mullerian inhibitory substance type II receptor [17]. Further, hormonal factors in BRCA1 mutation-associated OC development could be addressed by observing the effect of hormone treatment or reproduction on cancer incidence of heterozygous BRCA1 mutant mice. Recapitulation of breast cancers and OCs similar to those of mutation carriers could reveal targets and pathways of potential importance for treatment of BRCA1 mutation carriers.

The utility of a transgenic BRCA1 mutant mouse models has some limitations, however. Specifically for BRAT, interpretation would be complicated by the lack of a
mouse homolog to the human MMP1 gene. An animal model with a longer lifespan and
greater potential for stochastic mutation accumulation than the mouse would be
beneficial as well. The rabbit ovary may be useful for this endeavor, as the normal
histology of the rabbit ovarian surface has been described [18]. Further, in contrast to
humans, a bursal membrane surrounds the murine ovary, which potentially alters the
stromal/tumor microenvironment and acquisition of invasive and metastatic capabilities
by tumor cells.

In addition to animal models, mining previously collected epidemiologic data
could further support a role for BRAT or other BRCA1 mutants in development of OC.
For example, if mutation designation were available, other genetic alterations observed in
prophylactically removed ovaries or BRCA1 mutation-associated OCs could hint at
downstream targets of specific BRCA1 mutants or additional mutations that promote
tumorigenesis in carriers of that mutation.

Significance

The scope of importance of BRAT and its impacts are broad in the field of OC
pathology and treatment. Functions or targets of the BRAT protein may be common to
other similar BRCA1 mutants. The similarity of breast cancer to OC ratios for BRCA1
truncation mutants of similar sizes suggests BRCA1 mutants may share common
mechanisms of action. If there are common themes, this discovery could advance
understanding of and treatment of many high risk patients.

Information gleaned about BRCA1 mutation-associated OCs may also be applied
to sporadic cancers. Differences between sporadic and BRCA1 mutation-associated OC
are less well defined than for breast cancer. Unlike BRCA1 mutation-associated breast
cancers, which are frequently more proliferative and higher grade [19-21], reports disagree as to whether stage and grade differ significantly between BRCA1-associated and sporadic OC [22, 23]. In contrast to BRCA1-associated breast cancers, BRCA1 OCs lack a unique molecular profile compared to sporadic OCs [24, 25]. BRCA1 mutation-associated and sporadic OCs may share common downstream targets, such as Akt, c-Jun, or MMP1. Common signaling mediators shared between BRCA1 mutation-associated and sporadic OC increase the utility of drugs targeting these pathways by increasing the population of patients that will likely respond.

Most studies reveal that BRCA1-associated OCs are more frequently categorized as serous carcinomas and rarely as mucinous [23, 26]. The histologic subtypes of OC not only have different clinical impacts (ie treatment response and survival rates), but are thought to arise from distinct precursor regions, undergo distinct stepwise histologic changes, and occur though deregulation of distinct molecular pathways (Reviewed in [27]). Subtype-specific pre-malignant changes may be similar in BRCA1 and sporadic OCs. Indeed, surface invaginations and inclusion cysts, which are hypothesized to be the origin of some types of OC, occur more frequently in BRCA1 mutation carriers, but are present in the ovaries of the general population and increase with age [13].

Alternatively, BRAT’s mechanism of action may be unique, and understanding its contribution to OC and breast cancer risk may have limited application to prognosis and treatment of the general population. Regardless, increasing knowledge about BRAT will greatly benefit confirmed mutation carriers and patients through better risk assessment, decisions about prophylaxis and treatment, and treatment efficacy.
I have identified MMP1 as a novel target of the BRAT BRCA1 mutation in HOSE. I have determined that BRAT increases MMP1 gene expression and enhances cellular and secreted pro-MMP1 in a c-Jun dependent manner involving several AP1 sites in the MMP1 promoter. I have also determined that BRAT’s impacts on chemosensitivity and gene regulation are specific for the ovary. Taken together, these early molecular changes could poise cells for transformation or acquisition of invasive or metastatic ability. Further exploration of these changes can increase our understanding of early steps of OC development and help identify potential screening and treatment strategies.
References


About the Author

Rebecca Linger completed her undergraduate studies at West Virginia Wesleyan College, where she graduated with a B.S. degree in Biology and a minor in Chemistry. During this time, she received a Mary Babb Randolph Cancer Center Undergraduate Research Fellowship at West Virginia University, where she completed a summer research project. Rebecca received her M.S. in Molecular Physiology and Biological Physics from the University of Virginia, where she was awarded a Congressionally Directed Medical Research Programs Department of Defense Breast Cancer Research Predoctoral Traineeship. She was also invited to the American Association for Cancer Research Edward A. Smuckler Memorial Pathobiology of Cancer Workshop in Aspen, CO. Rebecca joined the Medical Science Ph.D. Program in the USF College of Medicine in 2007. She presented her research at the 2010 American Association for Cancer Research Annual Meeting in Washington, D.C. and at USF Health Research Day in 2009 and 2010, where she was awarded Outstanding Poster Presentation in 2010. Rebecca was active in the Association of Medical Science Graduate Students, and served as the association’s American Cancer Society Relay for Life Team Captain in 2009. Also in 2009, she was awarded the Association of Medical Science Graduate Students’ Dr. K. Student Leadership Award for outstanding leadership and service.