EXPRESSION AND FUNCTION OF THE TUMOR SUPPRESSOR CANDIDATE HTRA1 IN HUMAN BREAST EPITHELIAL CELLS

A Dissertation in

Genetics

by

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Doctor of Philosophy

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ABSTRACT

A number of studies have reported that expression of the serine protease HtrA1 is reduced or lost in many human primary cancers, including ovarian, endometrial, hepatocellular carcinomas and melanoma, compared to their non-tumorigenic surrounding tissues, suggesting that HtrA1 may function as a tumor suppressor. There are also observations indicating that breast, ovarian, and gastric tumors with higher levels of HtrA1 expression have a greater response to chemotherapies compared with those with lower HtrA1 levels. However, the precise mechanism(s) leading to the down regulation of HtrA1 during malignant transformation are unclear, and the putative tumor suppressor functions of HtrA1 in human cancers are poorly understood.

Recently, emerging data suggest that epithelial to mesenchymal transition (EMT) and cancer stem cells (CSCs) play critical roles in the regulation of resistance to chemotherapeutics. Acquisition of mesenchymal-like properties indicate a switch of cancer cells from proliferation to an invasive phenotype, which could facilitate escape from effects of drugs that target cell growth pathways, eventually leading to therapeutic resistance.

Therefore, we hypothesized that HtrA1 gene expression would be decreased in breast epithelial cancer cells/tissues, compared with their normal counterparts, and this down-regulation of HtrA1 might contribute to the drug resistance in cancer cells at least in part through induction of the EMT.

The overall goal of this thesis project was to test this hypothesis by: 1) characterizing HtrA1 gene expression in both normal and cancerous human breast
epithelial cells; 2) investigating potential functional mechanisms of HtrA1 in breast carcinogenesis in breast epithelial cells.

We characterized HtrA1 expression in seven human breast epithelial cell lines. Two of them were immortalized, non-tumorigenic “normal” breast epithelial cell lines whereas five were breast cancer cell lines. We found that HtrA1 expression was dramatically decreased in human breast cancer cell lines compared with their “normal” counterparts. The decreased HtrA1 expression was largely attributable to transcriptional defects, which involve epigenetic silencing mechanisms. A dramatically decreased expression of HtrA1 was also observed in human ductal carcinoma in situ (DCIS) lesions compared with “normal” breast epithelium. To further study the HtrA1 gene functions in breast epithelial cells, we modified the “normal” MCF10A cell line. We created 4 stably transfected cell lines expressing 4 siRNAs targeted to HtrA1 (HtrA1 knockdown cells), as well as a stably transfected cell line overexpressing HtrA1. We found that the HtrA1 knockdown cells underwent the EMT, acquiring a mesenchymal phenotype, encompassing increased growth rate, increased migration and invasion ability, expression of mesenchymal cell biomarkers, and decreased expression of epithelial cell biomarkers as well as decreased expression of microRNA 200 family members. Further, reduction of HtrA1 expression activated ATM and resulted in formation of γH2AX foci following acute oxidative stress, whereas overexpression of HtrA1 prevented formation of the foci.

Collectively, these results suggest that HtrA1 may function as an important tumor suppressor gene in breast cancer. The association of HtrA1 expression levels with resistance to chemotherapeutics might relate to the EMT, as well as to important effects
of HtrA1 in DNA damage response components. From this point of view, HtrA1 may serve as a new biomarker in breast cancer diagnosis and a therapeutic target and outcome predictor.
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LIST OF ABBREVIATIONS

AJ  adherin junction
bHLH  basic helix-loop-helix
bp  base pair
β  beta
BME  basement membrane extract
cDNA  complementary DNA
CLDN  claudin
CSC  cancer stem cell
ChIP  chromatin immunoprecipitation
°C  degree Celsius
DCIS  ductal carcinoma in situ
DDR  DNA damage response
DMSO  dimethylsulfoxide
DNA  deoxyribonucleic acid
DOX  doxorubicin
DSBs  double strand breaks
ECM  extracellular matrix
EMT  epithelial to mesenchymal transition
γ  gamma
GO  gene ontology
<table>
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<tr>
<td>HDAC</td>
<td>histone deacetylase</td>
</tr>
<tr>
<td>HPV</td>
<td>human papillomavirus</td>
</tr>
<tr>
<td>hr</td>
<td>hour</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>hydorgen peroxide</td>
</tr>
<tr>
<td>IGFBP</td>
<td>insulin growth factor binding protein</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>KI</td>
<td>kausal protease inhibitor</td>
</tr>
<tr>
<td>KRT</td>
<td>cytokeratin</td>
</tr>
<tr>
<td>LOH</td>
<td>loss of heterozygosity</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>µg</td>
<td>microgram</td>
</tr>
<tr>
<td>µl</td>
<td>microliter</td>
</tr>
<tr>
<td>µM</td>
<td>micromoles per liter</td>
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<tr>
<td>miR</td>
<td>microRNA</td>
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<tr>
<td>MW</td>
<td>molecular weight</td>
</tr>
<tr>
<td>ng</td>
<td>nanogram</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PTP</td>
<td>protein tyrosine phosphotase</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
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<tr>
<td>RT</td>
<td>room temperature</td>
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<tr>
<td>RTK</td>
<td>receptor tyrosine kinase</td>
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<tr>
<td>RT-QPCR</td>
<td>reverse transcription-quantitative realtime PCR</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>SS</td>
<td>signal sequence</td>
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<tr>
<td>ssDNA</td>
<td>single-strand DNA</td>
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<tr>
<td>TGFβ</td>
<td>transforming growth factor beta</td>
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<tr>
<td>TJ</td>
<td>tight junction</td>
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<tr>
<td>TSA</td>
<td>trichostatin A</td>
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ACKNOWLEDGEMENTS

Several years ago, I was led to this scientifically advanced country to pursue a Ph.D. degree in Genetics for my career. From then on, I started an exciting journey.

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Chapter 1

Thesis Introduction

Proteases play essential roles in multiple biological processes. Beyond their functions in protein catabolism, proteases can selectively cleave substrates and thus influencing cell behavior, survival, and death [1]. According to the MEROPS database, the complete set of proteases produced in the human proteosome, consists of at least 569 proteases distributed in five classes: 194 metalloproteinases, 176 serine, 150 cysteine, 28 threonine and 21 aspartic proteases [2].

For a long time proteases, especially extracellular proteases, are believed to be associated with tumor progression since they can degrade extracellular matrix, which facilitates cell migration and invasion. However, in recent clinical trials, patients treated with broad-range metalloproteinase inhibitors showed no effects, or even an acceleration of tumor growth [3, 4]. This puzzling finding suggested that some extracellular proteases might actually have anti-tumor properties.

Serine proteases, PRSS3 (also known as trypsinogen IV), PRSS8 (prostasin), and PRSS21 (testisin) were categorized as tumor-protective proteases in the human degradome [2]. PRSS11 (HtrA1) may represent another tumor suppressor within this same group.

HtrA1 has been shown to be down-regulated in various human solid tumors. Studies reported that hHtrA1 mRNA was absent or significantly down-regulated in ovarian cancer [5], and it was suggested that HtrA1 may have tumor suppressor
functions. A similar down-regulation was observed in melanomas [6]. Recently Mullany et al. revealed a complete loss of HtrA1 expression in all seven papillary serous endometrial cancer cell lines examined by Western blot analysis, as well as a low level of HtrA1 expression in primary endometrial cancer tumors by immunohistochemical analysis [7]. In another recent study, HtrA1 was found at greatly reduced levels in hepatocellular carcinoma specimens compared to adjacent normal liver tissues [8]. However, the mechanism(s) leading to the down regulation of HtrA1 during malignant transformation are unclear.

From clinical results, Chien et al. found that HtrA1 could modulate cisplatin- and paclitaxel-induced cytotoxicity and further found that primary ovarian and gastric patients with tumors expressing higher levels of HtrA1 showed a higher response rate to chemotherapies compared to those lower level tumors [9].

More importantly, Folgueira et al. a group of researchers found a trio of genes (PRSS11/HtrA1, MTSS1, CLPTM1) that could distinguish doxorubicin-responsive from non-responsive breast tumors with 95% accuracy [10]. However, the mechanism of action of HtrA1 in this therapeutic response is not understood.

Breast cancer is not a single disease, but rather is comprised of varied subtypes of different molecular features, which may associate with different clinic outcomes.

To date, many therapies and clinical trials have made progress on breast cancer treatment [11]. Nonetheless, much improvement is still needed. Many patients receiving systematic therapy for breast cancer either do not need it or will not benefit from it. This tells us that suitable biomarkers are required to determine whether the therapy is safe and
whether the therapy will be effective. Well-defined biomarkers will also be helpful for early diagnosis of breast cancer and might reduce mortality from this disease.

Therefore the overall objective of this thesis project was to answer two questions: 1) What are the gene expression profile and expression regulation mechanism(s) of HtrA1 in breast cells? 2) What are the potential mechanisms/functions of HtrA1 in breast carcinogenesis and drug resistance?

To answer the first question, initially, we started to characterize HtrA1 gene expression in a limited number of human breast tissue samples with immunohistochemistry analysis. Simultaneously, we evaluated the HtrA1 expression profile in multiple breast cell lines, including 2 immortalized non-tumorigenic breast cell lines and 5 carcinoma cell lines, by RT-PCR and Northern analysis. Moreover, we investigated potential epigenetic regulation of HtrA1 gene expression with promoter region DNA methylation bisulfite sequencing and the restoration of HtrA1 expression by decitabine (DNA methyltransferase inhibitor) and/or trichostatin A (TSA, histone deacetylase inhibitor) treatment (Results were shown in Chapter 3).

To examine the effects of HtrA1 expression levels in breast cells, we created 4 HtrA1 knockdown cell lines by stably expressing siRNAs, as well as an HtrA1 over-expressing cell line by stable transfection of the HtrA1 full length coding sequence in the immortalized non-tumorigenic breast cell line, MCF10A. In this model, we detected the EMT phenotype, including expressions of EMT biomarkers and increased cell migration/invasion ability, in HtrA1 down-regulated cells. We also compared the whole genome mRNA expression and microRNA expression profiles between HtrA1 knockdown or over-expressing cells vs the appropriate irrelevant controls.
Chapter 4 is the description of the creation of stable cell lines showing HtrA1 overexpressing or HtrA1 knockdown, and characterization of the positive clones and their corresponding HtrA1 expression levels. Further, whole human genome gene array was applied to the HtrA1 knockdown, overexpressing, and control cell lines.

In chapter 5, detailed studies of down-regulation of HtrA1 were performed, which showed induction of the EMT. Characterization of cell morphology and growth rate, cell migration and invasion ability, immunocytochemistry of EMT markers, microRNA profiles, and the involvement of the EGFR/AKT signaling pathway provided supportive data, which suggested a potential explanation for the relationship between decreased HtrA1 expression and drug resistance in human breast tumors.

In addition, to address the oxidative stress response function of HtrA1, we treated the HtrA1 knockdown and overexpression MCF10A cells with acute hydrogen peroxide and detected early DNA damage response events (Results were shown in Chapter 6).

The schematic view of this thesis proposal is shown in Figure 1-1.
Dramatically decreased in breast cancer cells, involving epigenetic silencing mechanisms (Chapter 3)

HtrA1

- Preventing EMT (Chapter 5)
- Protection from ROS-induced DNA damage (Chapter 6)

Figure 1-1: Schematic view of thesis proposal.
Chapter 2

Literature Review

1. HtrA1 and Its Biological Significance

A. HtrA1 History and Other Family Members

The serine protease HtrA is a protein quality control molecule. It was initially identified in E. coli by two mutant phenotypes. Mutants either did not grow at elevated temperatures (HtrA, High temperature requirement) [12] or failed to digest misfolded protein in the periplasm (DegP) [13].

The human HtrA1 gene was initially identified as being expressed in human fibroblasts but not expressed after transformation with SV40 [14]. It is located on chromosomal 10q26.2 with 9 exons encoding 458 amino acids; from N-terminus to C-terminus contains a signal peptide (SS), insulin growth factor binding domain (IGFBP), Kazal protease inhibitor (KI) domain, trypsin-like protease domain, and a PDZ domain [15]. Figure 2-1 is the summary of Homo Sapiens HtrA family members domain organizations.

To date, four human homologues of HtrA family have been identified: HtrA1 (Prss11, L56), HtrA2 (Omi), HtrA3 (PRSP) and HtrA4. These homologues each contains a highly conserved trypsin-like serine protease domain and one or two C-terminal PDZ domains [16].
PDZ domains are protein-protein interaction modules that often play a central role as organizers of multimeric signaling complexes [17, 18]. Binding substrates in this domain has been suggested as a switch for activating the protease activity of both bacterial and human HtrA family members [15, 19]. It has been reported that a synthetic oligopeptide (GM130Pep), which can bind to HtrA1 with a high affinity, stimulated mouse HtrA1 protease activity 3- to 4-fold, but did not stimulate the activity of an HtrA1 mutant lacking the PDZ domain [20].

In vitro, HtrA1 was shown to bind to a broad range of Tgfβ family proteins, including Bmp4, Gdf5, Tgfβs and activin in a GST-pulldown assay in mouse myoblast C2C12 cells [21]. In vivo, during mouse embryo development, HtrA1 was found being expressed in specific areas where Tgfβ family proteins have regulatory activities [21].
The human HtrA1 has been found ubiquitously expressed in normal human tissues. Luca and Falco et al. reported that high to medium HtrA1 expression was detected in mature layers of epidermis, in secretory breast epithelium, in liver, and in kidney tubules of cortex. They also found a higher protein level in the epithelium of proliferative endometrium, in contrast to secretory endometrium, which is almost completely negative for this protein [22]. These results suggest that HtrA1 may have various functions in different cell types and in different settings.

HtrA2/Omi is localized in mitochondria and is released to the cytoplasm in response to apoptotic stimuli. HtrA2 induces cell death, both in a caspase-dependent manner [23, 24] by interacting with the inhibitor of apoptosis proteins, as well as in a caspase-independent manner [25, 26] that relies on its protease activity.

Human HtrA3 (also called pregnancy-related serine protease) has two mRNA isoforms produced by alternative splicing and both of them show active protease activity [27]. Although it shares a high homology with HtrA1 in amino acid sequence (Figure 2), it has different organ distributions. HtrA3 is highly expressed in heart, whereas the HtrA1 expression is low. HtrA1 expression is much higher in brain-related tissues, compared with low expression of HtrA3. Placenta has high expression of both HtrA1 and HtrA3. However, HtrA1 expression in placenta is much higher than in any other organ, whereas HtrA3 expression in placenta is lower than in the ovary, heart, and foetal heart [27].
B. HtrA1 in Diseases

The HtrA family of serine protease appears to be involved in many biological mechanisms in mammals. Specific for HtrA1, it is implicated in osteoarthritis, age-related macular degeneration, Alzheimer’s disease, cell migration and invasion, tumor progression, and chemotherapy-induced cytotoxicity [28].

Recently HtrA1 has been reported to be absent or significantly down-regulated in a variety of cancers and during their progression, including ovarian [29], melanoma [6] endometrial [7] and hepatocellular [8] carcinomas, and it has been suggested that HtrA1 has tumor suppressor functions. For example, HtrA1 knockdown in ovarian cancer cell line SKOV3 promoted cell anchorage-independent growth, while overexpression of
HtrA1 in another ovarian cancer cell line (OV2O2) induced cell death [29]. A similar down-regulation was observed in melanomas, and stably overexpression of HtrA1 in a melanoma cell line inhibited cell proliferation as well as xenograft growth in vivo in nude mice [6].

Similar to DegP in E. coli in response to stress stimuli from environment, human HtrA1 is also involved in a general stress response pathway. This is supported by up-regulation of HtrA1 during osteoarthritis [30] and ageing [31]. Direct evidence for HtrA1 up-regulated in response to oxidative stress was provided by Zurawa-Janicka et al [32]. They found both HtrA1 mRNA and protein levels were significantly increased in hamster kidney within the first five hours of estrogen treatment, a treatment known to generate ROS, whereas during prolonged estrogenization (6 months), a paradoxical reduction of both HtrA1 mRNA and protein levels was observed.

C. Functional Models for HtrA1 Actions

In 2008 and 2009, a group of researchers reported HtrA1’s binding substrates and its role in cell migration and invasion. First Ajayi et al. found that ectopic expression of HtrA1 in an extravillous trophoblast cell line (HTR-8/SVneo) significantly attenuated its migration and invasive capabilities [33]. This inhibition was not dependent on HtrA1’s protease activity since both wild-type and a proteolytically inactive mutant HtrA1 could significantly (P<0.01) change these capabilities compared to controls.

As a future direction, various deletion constructs of HtrA1 would be helpful to elucidate which domain is the functional switch of HtrA1 in cell migration and invasion.
Secondly, the same group reported that HtrA1 was a microtubule-associated protein. Intracellular HtrA1 was localized to microtubules (MTs) in a PDZ domain-dependent manner. In vitro, purified HtrA1 promoted microtubule assembly. Exogenous expression of HtrA1 disrupted microtubules and targeted tubulins for degradation [34]. MTs are highly dynamic noncovalent polymers of α- and β-tubulins. Many tumor suppressors associate with MTs and impose their tumor suppressing roles by regulating MTs functions related to cell polarity, division, and motility. Such tumor suppressors are as APC [35], RASSF1A [36], and Dlg [37].

Overall, these data indicate a role for HtrA1 in control of cell motility.

In another report, stable knockdown of HtrA1 in human ovarian cancer cell lines (SKOV3 and TOV21G) attenuated anoikis, a critical barrier for tumor metastasis [38]. He et al. assumed this was due to activation of the EGFR/AKT pathway, since they detected that HtrA1 interacted with EGFR both on the cell membrane and in the nucleus, using immunoprecipitation and immunofluorescence assays. Growing in suspension culture, these authors also found that in SKOV3 cells, HtrA1 expression was automatically up-regulated, which inhibited the EGFR/AKT signaling pathway, resulting in increased cell death, whereas transient transfection of proteolytically-inactive mutant HtrA1 in SKOV3 cells did not show any inhibition of EGFR/AKT pathway or increased cell death. HtrA1 could contribute its metastasis-suppressing capacity through inhibition of EGFR/AKT pathway.
D. HtrA1 in Therapeutics

In the last few years, several studies have been performed to characterize gene expression profiles that might predict response to chemotherapy in primary breast cancers. In one such study, Folqueira et al. found that HtrA1 was one of a trio of genes (PRSS11, MTSS1, CLPTM1), which could correctly distinguish doxorubicin-responsive from non-responsive breast tumors in 95% of the samples analyzed [10]. They analyzed biopsy samples obtained before primary treatment with doxorubicin and cyclophosphamide and then extracted RNAs for gene array analysis.

Doxorubicin and cyclophosphamide are two of the most used anti-tumor drug worldwide. They are associated with an 80% positive response rate for reduction of primary breast tumor size [39].

In ovarian cancer, Chien et al. found that HtrA1 could modulate cisplatin- and paclitaxel-induced cytotoxicity in ovarian cancer cell line SKOV3 [9]. Down-regulation of HtrA1 attenuated the cytotoxicity, while overexpression of HtrA1 enhanced it. They also observed that HtrA1 expression level was increased by chemotherapeutic treatments including cisplatin, paclitaxel, doxorubicin, gemcitabine, and etoposide, both in SKVOV3 cells and the breast cancer cell line MCF7. More importantly, they found a correlation between HtrA1 expression level and primary ovarian and gastric tumors chemosensitivity in vivo. Patients with tumors expressing higher levels of htrA1 showed a higher response rate compared to those lower level tumors.

Cisplatin and paclitaxel are two chemotherapeutics that are widely used for solid tumors [40]. Usually the majority of patients response well to these two agents early in
treatment, but subsequently develop resistance. Many researchers believe the chemoresistance is the result of changes of the balance between cell survival and cell death pathways [41-43]. For example, anti-apoptotic proteins, Bcl-2, Mcl-1 have been observed to be elevated in tumors, which relapse [44, 45], while reduced expression of proapoptotic protein Bax was found in poor-responding breast tumors [46]. Therefore, HtrA1 might play functions in chemosensitivity through interacting with proteins that are involved in programmed cell death.

All these reports indicate that down-regulation of HtrA1 may play an essential role in resistance to chemotherapy, and thus HtrA1 might serve as a good therapeutic index to several agents in multiple cancers.

2. Breast Cancer and Breast Cancer Cells

A. Breast Cancer Introduction

Breast cancer is the leading cause of cancer-related death in women worldwide. Although significant improvements have been made in both diagnosis and treatment of breast cancer, there are still several clinical and scientific problems to be solved, including prevention, more specific diagnosis, therapeutic resistance, and tumor recurrence [47].

The initiation of breast cancer is thought to be due to genetic and epigenetic transforming events in a single cell, while subsequent tumor progression is driven by the
accumulation of additional genetic (or epigenetic) changes under selection pressures. It has been suggested that each BC subtype might have distinct tumor initiation and progression pathways, which may be driven by genetic mutations, epigenetic changes, and microenvironmental influences [48].

The pathological and clinical stages of ductal breast cancer progression start with ductal hyperproliferation, with subsequent evolution into in situ and invasive carcinomas, and finally into metastatic disease [49, 50] (Figure 2-3). Normal breast ducts are composed of basement membrane and a layer of luminal epithelial and myoepithelial cells. Stroma cells include leukocytes, fibroblasts, myofibroblasts, and endothelial cells. In in situ carcinomas, myoepithelial cell content is decreased, while stroma cells are increased. With genetic and epigenetic alterations, loss of myoepithelial cells and basement membrane results in invasive carcinomas, in which tumor cells can invade surrounding tissues and migrate to distant organs, leading to metastases. DCIS is thought to be a precursor of invasive ductal carcinoma based on molecular, epidemiological, and pathological studies [50, 51]. On the molecular level, genes found mutated with high frequency in breast cancer include BRCA1 (breast cancer 1), BRCA2 (breast cancer 2), TP53 (tumor protein p53), and factors involved in pathways of PI3KCA/AKT/PTEN, TP53, and NFκB signaling pathways.

Based on several recent large-scale studies, allele variants and single-nucleotide polymorphisms (SNPs) of certain genes are also tightly associated with breast cancer risks. Examples include FGFR2 (fibroblast growth factor receptor 2), TNRC9 (thymocyte selection-associated high mobility group box 9), MAP3K1 (mitogen-activated kinase kinase kinase 1), LSP1 (lymphocyte-specific protein), CASP8 (caspase 8), and TGFβ1.
On the other hand, cancers may not be only formed in the tumor epithelial cells. Their surrounding cells and the stroma may also contribute or facilitate tumor progression. Cells composing the microenvironment include myoepithelial and endothelial cells, fibroblasts, myofibroblasts, leukocytes, and other cell types. In vivo and in vitro studies have shown these cells, and ECM molecules, could modulate the tissue specificity of normal breast and the growth, polarity, and invasive properties of breast cancer cells [52, 53].

From this point of view, epithelial-mesenchymal interactions and transitions might be the very important settings for tumor initiation.

A piece of the supportive evidence is that chronic inflammation could increase cancer risk [54]; inflammation is primarily a stroma reaction.

Another clinical result demonstrated that a vaccine-based method to eliminate tumor-associated macrophages significantly decreased tumor growth and progression of
breast and other carcinomas [55, 56]. It has also been found that mesenchymal stem cells within tumor stroma promoted breast cancer metastasis [57]. All these data suggests a new therapeutic strategy by targeting tumor microenvironment.

However, contradictory findings exist when addressing the origins of tumor initiation. A potential way to clarify the role of stroma cells in tumorigenesis is to determine the molecular changes that occur in all major cell types from normal breast tissue, DCIS, and invasive carcinomas.

B. Breast Cancer Classification

Although there is some controversy, research suggests that breast carcinomas can be divided into at least four different phenotypes: (1) normal-like, with gene expression profiles similar to non-cancerous breast tissue; (2) luminal phenotype, with expression of epithelial markers such as E-cadherin, CK8, 18, and 19; (3) ER-/HER2+ phenotype, with overexpression of ERBB2; and (4) basal-like phenotype, with expression of EGFR, p63, CK 5/6, 14, and 17 [58-60]. Based on these criteria, normal-like breast cancer cell lines include SK-BR-7, Hs578, MDA-MB-435, MDA-MB-231, MDA-MB-157, and et al. Luminal subtype breast cancer cell lines have MCF7, BT483, MDA-MB-361, MDA-MB-415, SK-BR-5, and et al. MDA-MB-453, SK-BR-3, MDA-Mb-330 et al cell lines are HER2+ breast cancer cell lines, and MDA-MB-468, BT20, SUM149PT, HCC1937 et al are basal-like subtype breast cancer cell lines [61]. In general, the basal-like tumors have the worst prognosis while luminal-type tumors have the best prognosis. Approximately 15% of breast cancers are basal-like [62], and are typically referred to as triple-negative
phenotype; that is, estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor-2 (HER2) negative [63]. Others have suggested if one also includes positive expression of EGFR and CK5/6 to the triple-negative profile, this gives a more specific definition of basal-like breast cancer, and a more precisely predictable outcome [64].

After screening 479 invasive breast carcinomas and 12 carcinosarcomas using 28 different markers by tissue microarray-based immunohistochemistry, it has been reported that EMT occurs preferentially in human breast tumors with “basal-like phenotype” [65]. Up-regulated EMT markers included vimentin, N-cadherin, cadherin-11, smooth-muscle-actin, and the extracellular matrix remodeling proteins SPARC, laminin, and fascin. Decreased epithelial markers included E-cadherin and various cytokeratins. Supportive results were obtained in vitro using the MCF10A cell line, an immortalized non-tumorigenic breast cell line. In response to low cell density, MCF10A cells spontaneously underwent morphologic and phenotypic EMT-like changes. It exhibited a basal-like phenotype and shared many features with mesenchymal cancer cell lines [66-70].

3. Epigenetic Mechanisms in Gene Expression Regulation

A. Introduction

Epigenetics is defined as heritable changes in gene expression without changes in DNA sequences. Since epigenetic silencing processes are mitotically heritable, they can
play similar roles and undergo similar selective processes to those involving genetic alterations in the development of a cancer, and they might occur at a higher rate compared to mutations in somatic cells. The current focus on cancer-related gene silencing involves three interwoven mechanisms, DNA methylation, histone covalent modifications and nucleosomal remodeling.

The importance of DNA methylation in cancers is well established. Global hypomethylation and gene promoter region hypermethylation are proposed to be the common mechanisms underlying development of human cancer [71]. The majority of cancer-related DNA methylation studies are concentrated on CpG island gene promoter methylation because the CpG-island promoter methylation can permanently silence genes in mammalian cells. However, there is no rigorous establishment that cytosine hypermethylation in such promoters causally blocks gene transcription.

Another aspect in epigenetic changes is genome-wide histone modifications. For example, loss of acetylation at lysine 16 and trimethylation at lysine 20 of histone H4 is a common marker of human cancers [72]. Deacetylation and trimethylation of lysine 9 in histone H3 or lysine 27 in histone H3 also participate in silencing of genes [73].

A link between DNA methylation and histone modifications has also been demonstrated. Methylated cytosine could attract methylated-DNA binding proteins and histone deacetylases to the methylated CpG islands during gene silencing [74, 75].

More recently, a link between chromatin remodeling and DNA methylation or histone modifications has been found. For example, the catalytic component of the SWI/SNF chromatin remodeling complex, Brm, has been found to be associated with the
methylated DNA binding protein MeCP2 [76], and histone modifications were shown using the ATP-dependent chromatin remodeling machines [77].

Therefore, these three epigenetic processes might all be linked to each other to silence important genes in human cancers.

B. DNA Methylation

In eukaryotes ranging from plants to human, DNA methylation is found exclusively at cytosine residues. Vertebrate genomes are globally methylated with only CpG islands being unmethylated. A “CpG island” (CGI) is a DNA patch of approximately 1000bp, which is GC-rich in base composition (~65%) compared with the genome as a whole (~40%) [78]. CpG islands are normally unmethylated, whereas the sporadic CpG sites in the rest of the genome are normally methylated. However, this pattern gradually reverses during aging and carcinogenesis, with resultant increases in methylated CpG islands and a global loss of methylation [79].

There are two regular methods to examine DNA methylation status, enzyme digestion-PCR based analysis and bisulfite genomic sequencing. The first method is based on the inability of HpaII to cut methylated CCGG sequence while the methylation-insensitive isoschizomer of HpaII, MspI, can digest this sequence, PCR amplification is then to check whether there are products or not. Only a few methylation candidate sites can be tested in each PCR reaction by using this approach. On the other hand, bisulfite sequencing analysis is able to examine the genome-wide changes in cancer cells compared to normal cells. Briefly, first, genomic DNA is denatured with NaOH and
deaminated with sodium bisulfite to convert all unmethylated cytosines to uracils. The subsequent PCR reaction will then convert “U” into “T” in the PCR product, and researchers can compare the ratio of “T” versus “C” residues to estimate the methylation percentage.

Changes in gene expression by DNA methylation are heritable through the activity of DNA methyltransferase, which specifically modify CpGs [80]. Normal cells use this mechanism to establish and maintain normal cell type-specific gene expression. However, during tumorigenesis, this mechanism is somehow subverted in cancer cells to render tumor suppressor genes transcriptionally inactive.

DNA methylation is a reversible process. Three major DNA methyltransferases catalyze this process: DNMT1, which generally maintains DNA methylation patterns throughout each cell division [81], and DNMT3a and DNMT3b, which catalyze de novo methylation at previously unmethylated genomic sites [82].

C. Histone Modifications

The building block of chromatin is the nucleosome, which consists of 147 base pairs of DNA wrapped around a histone octamer, which is composed of two copies of each of the four core histones, H2A, H2B, H3, and H4. These histone domains are subject to posttranslational modifications, such as acetylation, methylation, phosphorylation, and ubiquitination [83].
A correlation between histone acetylation and increased gene expression was discovered many years ago [84]. Acetylation at the N-termini of core histones is believed to induce the local opening of chromatin structures.

Under physiological conditions, nucleosomal arrays condense into a more compacted and higher-ordered structure known as heterochromatin [85, 86]. At any given time thousands of genes need to be activated or repressed in a coordinated process, and the chromatin must be remodeled to permit these events.

Reversible histone acetylation is controlled by histone acetyltransferases, which act as transcriptional coactivators, and histone deacetylases (HDACs), which repress transcription [87].

D. Chromatin Remodeling

Chromatin remodeling involves histone modifying enzymes and ATP-dependent chromatin remodeling complexes [88]. Histone modifying enzymes recognize and covalently modify the histone tails by acetylation, methylation, phosphorylation, ribosylation and ubiquitination [89]. Then ATP-dependent chromatin remodeling complexes recognize these histone marks and make the DNA accessible to transcription, replication and DNA repair complexes via ATP hydrolysis [90]. Thus, in this manner, chromatin structure is actually dynamically involved in regulation of gene expression.

Recently, chromatin remodeling has been directly linked to the other two mechanisms of epigenetic modifications. In particular, chromatin structures could be modulated by DNA methylation [91], as well as histone post-translational modifications.
Chromatin remodeling complexes can also affect histone mobilization [93, 94], new histone deposition [95], and histone replacement [96].

Besides the interplay with DNA methylation and histone modifications, which could potentially contribute to epigenetic gene silencing in cancers, mutations within the remodeling complex itself can directly play a causative role in certain human cancers.

The ATPase-dependent remodeling complexes include SWI/SNF (mating-type switching/sucrose non-fermenting), NuRD (nucleosome remodeling and deacetylation), and CHRAC (chromatin accessibility complex). The SWI/SNF complex consists of at least nine proteins and is evolutionarily conserved. A core subunit of the SWI/SNF is SNF5, which is in all known variants of the complex and is required for remodeling activity of SWI/SNF [97]. An SNF5 gene mutation has been associated with inactivation of p21 and p16 pathways [98] and stimulation of cell cycle progression [97]. Mutations in SNF5 have also been linked to numerous human cancers. For example, biallelic inactivating mutations in SNF5 have been demonstrated in malignant rhabdoid tumors [99]. Loss of SNF5 was frequently detected in pediatric choroid plexus carcinomas [100]. The SWI/SNF complex has also been identified interacting with numerous tumor suppressor genes and oncogenes, such as Rb, BRCA1, c-myc, and MLL (mixed-lineage leukaemia) [97].
4. Epithelial-Mesenchymal Transition (EMT) and Drug Resistance

A. Introduction

During the last few years, EMT has emerged as a “hot spot” of clinical research. EMT is an evolutionarily conserved developmental process. Epithelial cells are defined as surface barrier cells with secretory functions having distinct apical versus basolateral polarity, which are established by adherens and tight junctions. Mesenchymal cells perform scaffolding or anchoring functions and have multifunctional roles in tissue repair and wound healing [101]. EMT is essential for developmental processes in gastrulation, neural crest formation, heart morphogenesis, and musculoskeletal system and craniofacial structure formation [102]. However, similar transition can also happen during the metastasis of epithelial tumors.

In primary tumors, cells with metastatic capability represent only a small subpopulation of cells located in particular areas of the tumor [103]. For instance, single cancer cells and/or small cancer clusters have been found in the invasive front of some tumors and this has been shown to have prognostic significance in breast cancer [104]. Interestingly, recent studies demonstrated expression of EMT markers in the invasive front of a number of tumors, suggesting that EMT might be important in local invasion [105-107]. Although reports identifying EMT cells in human tumors are relatively uncommon, it is recognized that cancer cells might only undergo partial EMT as a transient event (Figure 2-4) [108]. In this model, a small minority of cells of epithelial origin detach from the tumor mass, an event, which is induced by increasing contacts
with tumor stroma. These cells are less differentiated, less cohesive, and have a strong connection with surrounding stroma. It is hypothesized that after they invade adjacent tissues, intravasate into blood vessels (or lymphatics), and are transported to distant organs, they have to return into an epithelial state to be able to colonise. This process is named as mesenchymal to epithelial transition (MET), which is also a fundamental embryonic process.

**Figure 2-4: Spatial and Temporal Illustration of EMT.** Spatially, increased contacts with the tumor stroma induce EMT in a minority of cells, which could be disseminated from the primary tumor resulting in local invasion. Temporally, cells with EMT locally invade a tissue, intravasate into blood vessels and are transported to distant organs. At the site of metastasis, carcinoma cells extravasate and revert to an epithelial state, by a process named MET, to form a metastatic colony. Figure was slightly modified from [108].

Identification of the EMT opens a new perspective of cancer research and provides the possibility of new therapeutic development.
B. EMT Phenotypes

Typical features of the EMT include loss of cell-cell adhesion and apical-basal polarity, as well as the acquisition of a fibroblastoid motile phenotype. This involves down-regulation of epithelial phenotypic markers, such as E-cadherin, Claudins, ZO-1/3, cytokeratins, with up-regulation of mesenchymal phenotypic markers, such as Vimentin, N-cadherin, fibronectin, and MMP1/2 [108-112].

E-cadherin and N-cadherin are calcium-dependent cell adhesion molecules that mediate cell-cell adhesion and cell migration and tumor invasion. Previous study has shown that N-cadherin is up-regulated in more invasive and less differentiated breast cancer cell lines, which lack E-cadherin expression [113].

Over 90% of cancers are of epithelial origin. Therefore, adherens junctions (AJs) and tight junctions (TJs) are considered to have critical functions in regulating the microenvironment around epithelial cells since the combination of AJs and TJs have important functions in the formation of epithelial cell sheets and also in the formation of paracellular barriers [114]. While adherens junctions maintain the adhesion between adjacent cells, tight junctions provide a tight seal between neighboring cells. The cadherins interact with cytoplasmic catenins that link the cadherin/catenin complex to the actin cytoskeleton [115]. The transmembrane tight junctions are composed mainly of claudins (at least 24 members), occludins, ZO-1/2/3, JAMs (junctional adhesion molecules) and CAR (coxsackie and adenovirus receptor) [114]. The cytoplasmic domains of these factors are highly organized and associated with cytoskeletal scaffolding proteins. The key connection of this event is the PDZ-binding motifs of
claudins binding to the PDZ-containing membrane scaffolding proteins ZO-1/2/3 and MUPP1 [116].

Several classes of transcription factors are considered as E-cadherin repressors, including Snail family members (1, 2, 3), zinc finger transcription factors, ZEB1 (δEF1), ZEB2 (SIP1), bHLH factors such as E12/E47 (TCF3) and Twist, and recently identified FOXC2 (forkhead 1), HOXB7 (homeobox gene B7), CBF-A (NFYB), and KLF8 [102].

Emerging data suggest crosstalk among these EMT transcription factors, allowing them to form a signaling network that is responsible for establishing and maintaining mesenchymal cell phenotypes [117].

C. EMT Involved Mechanisms and Signaling Pathways

To date, the mechanisms we know that can activate EMT involve multiple extracellular triggers and intracellular signaling pathways, such as TGFβ signaling, microRNA-200 family members, Wnt signaling, Notch signaling, receptor tyrosine kinases (RTKs), hypoxia, and epithelial cell-stroma cell interactions, NFκB-dependent mechanisms, as well as steroid hormone signaling [111, 118-120]. Recently, the deregulation of handling of reactive oxygen species (ROS) has been related to EMT, which potentially represents a step towards tumor invasion and metastasis [121].

Wnt signaling may induce the EMT through inhibition of glycogen synthase kinase-3β (GSK3β)-mediated phosphorylation and associated degradation of β-catenin in the cytoplasm [111]. Numerous RTKs have been found mutated and constitutively active in many cancer types.
Using a breast cancer model overexpressing a constitutively activated p65 subunit of NFκB, Chua et al. demonstrated that NFκB induced an EMT that coincided with elevated expression of ZEB1 and ZEB2, with concomitant loss of epithelial-specific genes, such as E-cadherin and desmoplakin [122]. These data point to a role for NFκB signaling during breast cancer progression.

**a. TGFβ Signaling Pathway**

One predominant driver of the EMT is the pleiotropic cytokine transforming growth factor TGFβ1 [123]. The TGFβs are a family of pleiotropic cytokines and their diverse effects allow the TGFβs to play multiple (and even opposing) roles in different contexts during embryonic development, tissue homeostasis and cancer progression [124].

In normal development and homeostasis, the production of TGFβ is highly regulated. Via multi-step processes, mature active TGFβ dimmers can bind to cell surface TGFβ receptors and activate both canonical Smad-mediated, as well as Smad-independent, signaling pathways [125].

Normally, in developmental settings, TGFβ promotes cell cycle exit, through induction of p15 and p21, which are cdk inhibitors, with repression of c-myc [126]. During mouse mammary gland development, TGFβ inhibits ductal cell proliferation to maintain the normal branching morphogenesis of ductal structures in virgin mice [127].

While the TGFβ signaling is often mediating cytostasis in epithelial cells, in some developmental stage- and tissue-specific conditions, it actually promotes EMTs.
Examples include palatal fusion during cranio-facial morphogenesis [128], formation of the endocardial cushions during cardiac valve development [129] [129, 130], and wound healing. However, in human pathology, TGFβ has been identified as a main driver of fibrotic disease in organs such as heart, kidney, and liver [131-133]. In addition, TGFβ can also act in a paracrine manner within the tumor microenvironment, promoting tumor growth, through immune suppression and promotion of angiogenesis [134].

Wyatt and Khew-Goodall suggested that the transcription factor Pez is an inducer of TGFβ signaling [124]. Exogenous expression of Pez in epithelial MDCK cells prompted an EMT phenotype through activation of TGFβs.

PTP-Pez (also known as PTPD2 or PTPN14) is a non-receptor protein tyrosine phosphatase (PTP), which has been found localized to multiple subcellular positions, depending on factors such as cell type, cell-matrix adherence, serine phosphorylation, and cell confluency [135-137]. Recently, a number of mutations in Pez have been shown to be associated with breast and colorectal cancers [138, 139].

**b. MicroRNAs**

Another component involved in the EMT are microRNAs (miRNAs) with specific miRNAs regulating the expression of genes important in the EMT. MiRNAs are small non-coding RNAs which to play important roles in a broad range of biological processes, including development, apoptosis, cell cycle progression, cellular proliferation, and cancer initiation and metastasis [140]. Approximately 30-50% of miRs are encoded within the introns of protein coding genes and the remaining miRs are located in
intergenic sites [141]. They may account for 2-3% of total number of human genes, but potentially regulate at least 30% of those genes [142, 143]. Each miRNA is predicted to have many targets and every mRNA may be regulated by multiple miRNAs [144-146].

The biogenesis of miRNAs has only been elucidated in recent years. Since many of them are located in the introns of protein-coding genes and are generally transcribed by Pol II, their expression can just be spatially and temporally regulated similar to other protein encoding genes [145]. However, many miRNAs have their own promoters. The microRNA pathway begins with endogenously encoded primary microRNA transcripts (pri-miRNAs) transcribed by RNA polymerase II (Pol II) or PolIII. They are processed by the Drosha enzyme complex within the nucleus to produce precursor miRNAs (pre-miRNAs). These precursors are transported from the nucleus and then bind to the Dicer enzyme complex in the cytoplasm. When the RNA duplex loaded onto RISC has imperfect sequence complementarity, the passenger strand is unwound leaving a mature miRNA bound to the active site of RISC. The mature miRNA then recognizes target sites in the mRNA, leading to direct translational inhibition.

The first link between miRNAs and cancer came from the discovery that they are frequently located in “fragile sites”, cancer-associated genomic regions including regions of amplification, loss of heterozygosity, breakpoint regions in or near oncogenes and tumor suppressor genes [147].

O’Day and Lal (2010) summarized the literature for microRNAs involved in breast cancer. Several tumor suppressor miRNAs (miR-206, miR-17-5p, miR-125a, miR-125b, miR200, let-7, miR-34, and miR-31) are lost and certain oncogenic miRNAs (miR-
miR-155, miR-10b, miR-373 and miR-520c) are overexpressed in breast cancers [148].

Gregory et al. (2008) found that all five microRNA-200 family members (miR-200a, miR-200b, miR-200c, miR-141, and miR-429) and miR-205 were dramatically down-regulated in cells that had undergone the EMT in response to TGFβ, or ectopic expression of PTP-Pez, and overexpression of the miR-200 family was sufficient to prevent TGF-β-induced EMT [118]. Lliopoulos et al. (2009) showed that microRNA200 family members were differentially regulated by Akt isoforms. Decrease in the ratio of Akt1 versus Akt2 in cells induced the down-regulation of miR-200, and promoted the EMT and a stem cell-like phenotype [119]. The Clarke lab has shown that mammosphere-forming activity is abrogated in both normal and malignant mammary stem cells when the EMT program is shut down by overexpression of miR200c [149].

MicroRNAs may be able to more rapidly to change gene expression in response to environment stimuli compared to protein coding genes, since they do not need to be translated after transcription to exert their effects.

c. Hedgehog Signaling

Hedgehog family members are major regulators of embryogenesis, adult tissue homeostasis, and carcinogenesis [150]. Hedgehog signals can directly up-regulate CCND1 and CCND2 for cell cycle acceleration, FOXA2, FOXC2, FOXE1, FOXF1, FOXL1, FOXP3, POU3F1, RUNX2, SOX13, and TBX2 for cell fate determination, JAG2, INHBC, INHBE, and SFRP1 in stem cell signaling network [151]. Hedgehog
signals can also crosstalk with WNT, EGF/FGF, TGFβ, Notch signaling pathways and microRNA regulatory networks, which are implicated in EMT [151].

d. Angiogenesis

Growth of solid tumors, including breast, lung and prostate carcinomas, are considered dependent upon angiegenesis. Rapid cell growth and proliferation result in a dramatic surge of oxygen demand. Therefore, tumor tissues over consume oxygen supplies and thus suffer from hypoxia. Tumor angiogenesis is often mediated by hypoxia secondary to tumor growth or increased oncogenic signaling [120]. Both mechanisms result in increased hypoxia-inducible factor-1 alpha (HIF-1α) signaling and its transcriptional target vascular endothelial growth factor (VEGF).

HIF-1 has a key role in cellular response to hypoxia, including the regulation of genes involved in energy metabolism, angiogenesis and apoptosis [152]. HIF-1 is a heterodimeric transcription factor that is composed of HIF-1α and HIF-1β. Both subunits belong to the basic-helix-loop-helix (bHLH) superfamily of eukaryotic transcription factors [153]. Elevated HIF-1α expression is common in breast cancers and its expression levels are highly correlated to increased vascularisation, tumor invasiveness and poor clinical prognosis [153]. Under normoxic conditions, HIF-1α levels are tightly controlled by pVHL-mediated degradation, as well as p53-targeted and forkhead transcription factor FOXO4-induced proteolysis, but they are stabilized by hypoxia [152].

Cannito et al. (2008) suggested that hypoxia-induced EMT might process through a serial of events including phosphorylation and inactivation of GSK-3β, SNAIL and β-
catanin nuclear translocation, E-cadherin loss of function, and a significant increase in chemotaxis and invasiveness. Eventually, the migration and invasiveness are sustained by HIF-1α and vascular endothelial growth factor (VEGF) [154].

e. EGFR/AKT Pathway

Normal cell growth is tightly regulated by communications between growth factors and their receptors through which external signals can be transduced into intracellular compartments.

The human epidermal growth factor receptor family comprises four members: EGFR (HER1, ERBB1), EGFR2 (HER2, ERBB2), HER3 and HER4. They are transmembrane glycoproteins containing an extracellular ligand binding domain and an intracellular receptor tyrosine kinase (RTK) domain. Binding of ligand growth factors, such as epidermal growth factor (EGF), transforming growth factor (TGF), amphiregulin (AR), epiregulin (ER), neuroregulin (NR), to EGFR, HER3, and HER4 induces receptor dimerization and activation of intracellular protein tyrosine kinase (TK) with subsequent initiation of numerous downstream signaling pathways [155] (Figure 2-5).
The serine-threonine kinases Akt family is composed of three members: Akt1/2/3 [156]. Akts are the downstream mediators of phosphatidylinositol 3’ kinase (PI3K), and through the phosphorylation of their substrate targets, Akts impact on numerous cellular processes [157].

Emerging data shown that Akt1 and Akt2 may play different, even opposing, roles in tumor initiation and progression. For example, in one study of knockdown of Akt1 using shRNA in the MCF10A cell line dramatically increased cell migration [158]. In contrast, Akt2 down-regulation did not affect migration, and the combination of down-
regulation of both Akt1 an Akt2 abrogated the migration effect of Akt1 knockdown, which indicated that Akt2 is somehow required for the migration phenotype.

As noted earlier, Lliopoulos et al. showed that microRNA200 family members were differentially regulated by Akt isoforms. Decrease in the ratio of Akt1 versus Akt2 in cells induced the down-regulation of miR-200, which promoted the EMT and a stem cell-like phenotype [119].

D. EMT and Cancer Stem Cells in Drug Resistance

Chemotherapy is obviously an important strategy for cancer treatment. However, intrinsic and acquired chemoresistance can commonly cause tumor recurrence. The differentiation state of a tumor may contribute de novo resistance and the chemotherapy itself could promote the development of drug resistance though multiple mechanisms, such as accelerated drug efflux, drug inactivation, alterations in drug target, processing of drug-induced damage, and evasion of apoptosis [159].

Recently emerging data suggest that the EMT as well as cancer stem cells (CSCs) play critical roles in regulation of drug resistance [108, 112, 121, 160, 161]. Direct evidence showing that EMT might be associated with chemoresistance, was provided by Creighton et al. (2009). They found that the residual breast tumor cell populations surviving after conventional endocrine therapy or chemotherapy displayed both tumor-initiating and mesenchymal features [162].
The EMT has also been implicated in drug resistance in several other studies. In urothelial carcinoma cell lines, silencing of E-cadherin expression was associated with cetuximab (EGFR inhibitor) resistance [163]. Gemcitabine (a DNA synthesis inhibitor)-resistance in pancreatic cells with higher invasive capacity [164] and oxaliplatin (DNA cross-linker)-resistance in colorectal cancer cells [165] have also been associated with the EMT. In addition, EMT has been implicated in Lapatinib (a HER2 and EGFR inhibitor) resistance in breast cancer [166] and paclitaxel (microtubule dynamics suppressor) resistance in epithelial ovarian carcinoma [167].

Each patient’s tumor is a heterogeneous population of cells, so that within the tumor a small subpopulation of cells have greater tumorigenic potential than others [168]. Those with tumorigenic potential cells are referred as cancer stem cells (CSCs). CSCs are often compared with normal stem cells. Both cells are multipotent or pluripotent progenitor cells that can self-renew and divide by asymmetric cell division to give rise to differentiated or committed progenitors [169]. However, CSCs are fundamentally different from normal stem cells in several aspects. For instance, normal stem cells are controlled for their proliferation and are maintained for genomic integrity, whereas cancer stem cells are usually out of control of these processes [170].

Depending on the microenvironment, CSCs could arise from normal stem cells or from dedifferentiation of differentiated cells to a stem cell-like state [171]. In an early study, Bonnet and Dick isolated the CD34+/CD38- leukemia-initiating cells in acute myeloid leukemia. Those cells could form large colonies in immuno-compromised SCID mice and exhibit self-renewal and pluripotent properties, which were ascribed to normal stem cells [172]. This is suggesting that CSCs might come from normal stem cells.
However, differentiated cells can also be induced to obtain the pluripotency of embryonic stem cells [173, 174], and those induced pluripotent stem cells were able to form teratomas in SCID mice.

In recent studies, tumor-initiating cells with CSC signatures have been isolated by expression of distinct cell surface markers in multiple solid malignancies. For instance, CD44\textsuperscript{high}/CD24\textsuperscript{low} was identified as breast cancer-initiating cell markers [168] and aldehyde dehydrogenase 1 (ALDH1) was another specific marker for breast CSCs [175]. CD133+ tumor-initiating cells were then isolated from colon cancers [176] and pancreatic cancers [177]. In addition, epithelial cell adhesion molecule (EpCAM) was found as a CSC marker in hepatocellular carcinomas [178].

It has been reported that EMT traits in human breast cancer cell lines paralleled the CD44\textsuperscript{high}/CD24\textsuperscript{low} stem cell phenotype in human breast cancer [110]. Moreover, gene products that could distinguish Basal B from Basal A and Luminal cell lines showed concordance with those defined breast CSCs isolated from clinical samples [179]. It has also been found that the CD44\textsuperscript{+}/CD24\textsuperscript{flow} subpopulation of cells was more resistant to chemotherapy in paired primary human breast cancer biopsies [180]. Therefore, understanding of the cellular origin and the molecular mechanisms of EMT and CSCs will definitely benefit the future chemotherapeutics and the whole cancer research world.
5. Oxidative Stress and DNA Damage Response

A. Introduction of Reactive Oxygen Species (ROS)

Oxidative stress is commonly termed as the cumulative production of reactive oxygen species (ROS) along with reactive nitrogen species (RNS) through either endogenous or exogenous insults [181]. They both have deleterious and beneficial effects. From one side, more and more evidence shows that ROS can act as secondary messengers in intracellular signaling pathways, and thus chronic ROS may induce the oncogenic phenotype of cells. However, ROS can also induce cellular senescence and apoptosis and therefore have an anti-tumorigenic characteristic.

Sources of ROS and RNS include those generated during irradiation by UV light, by X-rays and by gamma-rays; products of metal-catalyzed reactions; pollutants in the atmosphere; those produced by neutrophils and macrophages during inflammation; by-products of mitochondria-catalyzed electron transport reactions and other mechanisms [182]. In order to balance the effects of ROS and RNS, antioxidant reactions are extremely important for defense systems, which perform by both enzymatic and non-enzymatic antioxidants. The most efficient enzymatic antioxidants include: superoxide dismutase (Cu, Zn-SOD; Mn-SOD), catalase, glutathione peroxidase [183]; and non-enzymatic antioxidants involve: Vitamin C, Vitamin E, carotenoids, thiol (glutathione, thioredoxin, lipoic acid), flavonoids, selenium [184].

Oxidation and reduction reactions in biological systems are called redox reactions. ROS can influence the redox status of cells to cause either a positive response
(cell proliferation) or a negative response (growth arrest or cell death). High concentrations of ROS generally cause cell death or even necrosis, whereas low concentration of ROS can stimulate proliferation and survival in a wide range of cell types [181].

ROS play an important role as second messengers in signal transduction [185]. In addition to causing direct DNA damage, ROS can also activate cytokines and growth factor signaling, non-receptor tyrosine kinases, Ras, proteintyrosine phosphatases, serine/threonine kinases, and several transcription factors, such as AP-1, NF-κB, p53, and NFAT and HIF-1 (Figure 2-6).

**Figure 2-6**: ROS-induced signaling pathways. ROS can stimulate several signaling cascades to lead to the activation of redox-regulated transcription factors (AP-1, NFXB, HIF1, NFAT, p53), such as cytokines and growth factor signaling, Ras, protein tyrosine phosphatases, serine/threonine kinase. In addition, ROS can directly
B. Antioxidant and DNA Damage Response

As one of the normal cellular metabolism products, ROS, which include superoxide, hydrogen peroxide, and hydroxy radical, can accumulate and damage proteins, lipids, and DNA [186]. In fact, it is suggested that accumulation of ROS-induced damage is a causal reason for the development of diseases such as aging and cancer [187]. In order to maintain a functional cellular environment, redox enzymes and DNA repair proteins need to dissipate ROS and fix the damages. Glutathione represents the major cellular redox buffer and therefore is a representative indicator for the redox environment of the cell [181]. Other important intracellular redox buffering capacity maintainers include: 1) Cu/Zn superoxide dismutase (SOD1), which can convert superoxide to hydrogen peroxide, and then can be turned into H$_2$O [188]. 2) thioredoxin (Trx), thioredoxin reductase (TrxR) and apurinic/apyrimidinic endonuclease 1/redox factor 1 (Ape1/Ref1), and protein disulfide isomerase (PDI) [189-192].

It has been shown that oxidative stress proteins form an interactive network, which collectively regulates oxidative stress (Figure 2-7). One of the by-products of redox metabolism is the hydroxyl radical (OH’), which can directly cause DNA lesions such as 8-oxoguanine (8-OxoG). Such lesions need to be recognized and removed by DNA glycosylases, leaving abasic sites. The DNA repair enzyme Ape1/Ref1 recognizes
abasic sites and cleaves the adjacent DNA backbone to let the base excision repair process continue [193].

Figure 2-7: Role of OxS and DNA repair proteins in cells. NM23-H1 induced DNA nicks can lead to DNA repair or apoptosis. Alkylating agents caused DNA lesions, such as 3-methylguanine (3-MeG), are recognized and removed by MPG. Ape1/Ref-1 then recognizes the apurinic sites and cleaves the DNA backbone. DNA repair can be completed by base excision repair pathway. If not repaired, DNA double-strand breaks may occur. γH2AX will be associated with the break site and recruit more DNA repair proteins. ROS include superoxide, hydrogen peroxide and hydroxyl radical, which are byproducts of normal cellular metabolism. They can cause DNA lesions such as 8-OxoG and peroxynitrite (OONO'). TrxR uses NADPH to reduce Trx, which in turn reduces and activates Ape1/Ref1. Activated Ape1/Ref1 then can reduce quite a few proteins. Figure was slightly modified from [186].

However, if abasic sites are accumulate, DNA double-strand breaks may occur. DNA double-strand breaks (DSBs) are the most severe genotoxic lesions. In responding
to DSBs, mammalian cells activate a series of proteins involved in signaling and repair pathways. DNA damage response is a highly coordinated pathway comprising a three-tiered signaling cascade as damage sensors, signal transducers, and downstream effectors [194]. Cells unable to respond to those breaks will die or potentially be predisposed to genomic instability and tumorigenesis.

C. Oxidative Stress and Carcinogenesis

ROS can affect a number of cellular processes important in tumor development. For example, ROS can modulate the cell cycle protein p53 [195] as well as ATM [196]. ROS caused by chronic inflammation can trigger tumor initiation through inhibition of apoptosis [197]. If oxidative DNA damages are not repaired before DNA replication, DNA mutation, replication error, and even genomic instability can occur.

a. Genetic and Epigenetic Changes Caused by ROS

Growing evidence suggests that ROS-induced oxidative stress could contribute to malignant transformation with both genotoxic (changing genomic DNA sequences leading to mutations) and non-genotoxic (modulating gene expression without directly affecting DNA) mechanisms [71].

Genetically, ROS can directly cause DNA damage. 8-hydroxy-2-deoxyguanosine (8-OHdG) is the most characterized DNA lesion [198]. ROS can also cause DNA single- or double-strand breaks, as discussed.
From an epigenetic point of view, DNA lesions caused by ROS can inhibit the DNA methyltransferases (DNMTs) binding to those DNA, thus resulting in global hypomethylation [199]. From another perspective, many major antioxidant enzymes have been shown epigenetically silenced in certain cancers. For example, manganese superoxide was found to exhibit decreased expression in human breast cancer [80] and pancreatic carcinoma [200], and its silencing was related to DNA hypermethylation. In addition, both quinone oxidoreductase 1 (NQO1), and glutathione S-transferase P1 (GSTP1) genes have been shown to be down-regulated via promoter hypermethylation in hepatocellular carcinoma [201], breast [202] and prostate [203] cancers.

**b. Acute and Chronic Oxidative Stress**

In recent years, growing evidence has shown the links between chronic cell stress and different kinds of diseases including cancer, cardiovascular disease, diabetes, and neurodegenerative disorders [204-207]. Actually, both acute and chronic stress can induce genetic and epigenetic changes in cells. But when adaptive pressure is taken into consideration, chronic stress presents a more severe challenge to the self-renewal properties of cellular systems.

More and more researchers now support the idea that survival adaptation mechanisms may explain the pre-malignant stages of cancer in which cells may attempt to maintain tissue homeostasis through increasing their numbers [208-211]. For example, in several cell systems, stem cell numbers increase with age but become less efficient in long-term self-renewal and their capacity to commit to normal cells.
Recently, Johnstone and Baylin addressed that epigenetic alterations could be attributed to acute and chronic stresses, especially chronic stress, which could be able to establish a persistent abnormal cell state even if the exposure is removed or diminished. Such memory in cells may eventually lead to disease pathology [212].

As a model for studying chronic oxidative stress, Benbrahim-Tallaa et al. used the toxic metal, cadmium, to malignantly transform MCF10A cells into a cell line with a basal-like phenotype [213]. In this regard, ROS are considered as crucial reasons for the cadmium-triggered tissue injuries [214].

Most researchers agree that important risks for cancers, including breast, colon, prostate, and lung malignancies, are inflammation and aging, both of which produce chronic oxidative exposure and increased DNA damage [215-217].

In Dr. Weisz’s lab, they found that even “normal” human mammary epithelium and surrounding stroma are under oxidative stress by using the 4-hydroxy-2-nonenal (4HNE), a reactive electrophilic breakdown product of lipid hydroperoxides, as the marker of chronic OxS. Samples were from young women reduction mammoplastics, yet many of them already show a high level of ROS. Taking latency into consideration, breast cancer initiation could be starting at a very young age.
Chapter 3

Characterization of HtrA1 Gene Expression in Human Breast Epithelial Cells

1. Introduction

The goal of this study was to examine HtrA1 gene expression and its regulation in human breast cancers.

A limited number of human breast tissue samples (normal ductal epithelial, ductal carcinoma in situ, and invasive cancer samples) were first characterized for HtrA1 protein levels with immunohistochemistry (HIC) analysis.

Simultaneously, multiple human breast epithelial cell lines were characterized for HtrA1 gene expression and expression regulation. The cell lines used in this study are summarized as below (Table 3-1).

MCF10A and MCF12A cells are two immortalized non-tumorigenic breast epithelial cell lines, whereas all the other five are breast epithelial carcinoma cell lines. We first examined HtrA1 mRNA levels by QPCR analysis and confirmed its expression with Northern blot analysis. We further investigated HtrA1 protein levels in all seven breast epithelial cell lines with Western blot analysis.

Our overall results indicated that HtrA1 gene expression was dramatically reduced in breast cancer tissues and cell lines compared with non-tumorigenic ones.
As known, loss of a gene expression during the development of cancer could be the result of numerous genetic and epigenetic changes. Genetically, it could involve mechanisms such as point mutations, loss of heterozygosity (LOH), and homozygous deletions. Epigenetically, it could involve three inter-related mechanisms including promoter DNA hypermethylation, histone deacetylation, and chromatin remodeling [218]. To exclude the possibility of HtrA1 gene mutations in the breast cancer cell lines, we accessed gene copy number, and then sequenced the coding region from each mRNA in all seven cell lines.

Aberrant cytosine methylation has been linked to altered gene expression in human tumors [219, 220]. The CpG islands located at promoter regions are usually unmethylated in normal tissues, but could potentially be methylated during normal aging

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Cell Type</th>
<th>Disease</th>
</tr>
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<tbody>
<tr>
<td>MCF10A</td>
<td>Epithelial ER-</td>
<td>Fibrocystic disease, Non-tumorigenic</td>
</tr>
<tr>
<td>MCF12A</td>
<td>Epithelial ER+</td>
<td>Non-tumorigenic</td>
</tr>
<tr>
<td>MCF7</td>
<td>Luminal, Epithelial ER+</td>
<td>Adenocarcinoma</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>Normal-like, Epithelial ER-</td>
<td>Adenocarcinoma</td>
</tr>
<tr>
<td>MDA-MB-468</td>
<td>Basal-like, Epithelial ER-</td>
<td>Adenocarcinoma</td>
</tr>
<tr>
<td>NM2C5 (derived from MDA-MB-435)</td>
<td>Normal-like, Epithelial ER-</td>
<td>Ductal carcinoma</td>
</tr>
<tr>
<td>M4A4 (derived from MDA-MB-435)</td>
<td>Normal-like, Epithelial ER-</td>
<td>Ductal carcinoma</td>
</tr>
</tbody>
</table>
or under pathological conditions. Traditionally, DNA methylation status can be detected on individual gene basis applying such as bisulfite sequencing and methylation-sensitive restriction enzyme digestion. Recent development enables DNA methylation analysis in a genome-wide scale [221, 222]. To test the involvement of DNA methylation changes in HtrA1 gene silencing during malignant transformations, we examined the HtrA1 promoter region DNA methylation status using bisulfite sequencing analysis.

From the literature, HtrA1 gene has also been shown negatively regulated by histone deacetylase 1 (HDAC1) in a model of HDAC1 knockout mouse embryonic stem cell [87].

Although the epigenetic mechanisms for silencing a gene are mitotic heritable, especially DNA methylation, it is dependent upon the activity of DNA methyltransferases and histone deacetylases functioning in the cells. They are reversible processes [87, 223]. Decitabine is a DNA methyltransferase inhibitor and trichostatin A (TSA) is a histone deacetylase inhibitor, both of which has been used to characterize the role of DNA methylation and histone deacetylation in controlling gene expression. Therefore, to further investigate if down-regulation of HtrA1 gene expression in breast cancer cell lines was regulated by epigenetic modifications, breast cancer cell lines were treated with either the DNA methyltransferase inhibitor decitabine, or the histone deacetylase inhibitor TSA or the combination of decitabine and TSA.
2. Materials and Methods

A. HIC for Human Breast Tissues

Tissue sections were deparaffinized and hydrated through xylenes and graded alcohol series and then were rinsed with PBS. For quenching endogenous peroxidase activity, the sections were incubated for 30 min in 0.3% H₂O₂ in dH₂O and then washed with PBS 3x 5 min and blocked with 2.5% normal horse serum for 30 min at RT. Sections were incubated with primary antibody HtrA1 monoclonal antibody from R&D systems (Cat. No. MAB2916, 1:500 dilution) at 4 °C overnight. The following day slides were washed for 5 min in PBS and ImmPress Reagent anti-mouse Ig from Vector (Cat. MP-7402) was added for 30 min at RT. The last chromagen was Vector VIP (Cat. SK-4600). Slides were then mounted for microscopy.

B. QPCR for HtrA1 in Breast Cells

Total RNAs from MCF10A, MCF12A, MCF7, MDA-MB-231, MDA-MB-468 NM2C5, and M4A4 cells (from ATCC) were extracted with Qiagen RNeasy mini kit (cat. #74904). Quantitative real-time PCR was performed with the QuantiTect Probe RT-PCR kit (Qiagen, Cat. # 204443) on Stratagene Mx4000 QPCR systems (Agilent Technologies). All quantification data were normalized to TBP, which acts as an internal control. The following primers and probe were used for human HtrA1, 5’-TTGTTTCGCAAGCTTCCGTT-3’ (forward), 5’-ACGTGGGCATTTGTCA-3’ (reverse), 5’-FAM-TCTAAACGAGAGGTCCGGT-CGTAGT-BHQ-3’ (probe); for human TBP, 5’-CACGGCAGATTTGCAGTTCT-3’ (forward), 5’-
TTCTTGCTGCCAGTCTGGACT-3’ (reverse), 5’-HEX-
TGTGCACAGGAGCCAAGAGTGAAGA-BHQ-3’ (probe).

Samples were collected from 3 independent harvests and results were expressed as means ± SE (standard error).

C. Northern Blotting Analysis

Total RNA for each cell line was extracted with Qiagen RNeasy mini kit (cat. #74904). For Northern analysis, we use NorthernMax-Gly kit from Ambion, #AM1946.

15µg of RNA for each sample was resolved on 1% agarose denaturing gel. Then RNAs were transferred to MAGNAgraph membrane (Osmonics, Inc. cat. # NJ0HYA0010) and sequentially probed with HtrA1 1.5kb full-length cDNA probe and 2.1kb β-actin cDNA probe as an internal control. Both probes were labeled with DECAprime II kit (#AM1456). The blot was exposed to X-ray film to develop. Experiments were performed with three independent biological samples for each cell line.

D. gDNA PCR for 7 Breast Cell Lines

20ng of genomic DNA from each cell line was used as template to amplify a 500bp region of HtrA1 gene, which covers its first exon by PCR reactions. The primers for HtrA1 gene are: 5’-TTGTTCGCAAGTAAAGAGA-3’ (forward), 5’-
ACGTGGGCATTTGTCACGATA-3’ (reverse). A single-copy gene for the Factor IX human blood clotting protein (access # K02402), which is located on the X-chromosome, was amplified in the same reaction as HtrA1 gene as loading control (Bioo Scientific,
Experiment was carried out with 24, 26, 28, and 30 cycles to avoid of the saturation of PCR amplification.

E. HtrA1 Coding-Sequence Sequencing for 7 Breast Cell Lines

It is starting from reverse transcribing 1µg total RNA for each cell line with SuperScript III reverse transcriptase kit (Invitrogen, #18080-093). Then amplify HtrA1 gene 1.5kb coding region sequence by PCR with HotStarTaq DNA polymerase and primers of 5’-CAGAGTCGCCATGCAGATCC-3’ (forward), 5’-GAA GTC CAG CTC ATG CCT CTG-3’ (reverse). Clone PCR products into TOPO vector (Invitrogen, #K4500-01SC) and check clones with EcoRI enzyme digestion. Then sequencing correct clones for each cell line and blast with NCBI genbank reference NM_002775.3. More than three colonies were sequenced for individual cell line.

F. HtrA1 Promoter Region DNA Methylation Study

The promoter sequence for the human HtrA1 gene was obtained from the UCSC Genome Bioinformatics website (www. genome.ucsc.edu). The possible transcription factor binding sites were examined in the 2000bp promoter sequence before transcription start site using the TRANSFAC database (www.gene-regulation.com/databases.html).

EpiTech Bisulfite kit from QIAGEN (cat. # 59104) was used for complete bisulfite conversion and cleanup of DNA for methylation analysis. Briefly, 1µg genomic DNA was incubated with sodium bisulfite buffer in a thermo cycling condition for 5hr. After the incubation, converted DNAs were cleaned up with the kit. The cleanup products were directly used for PCR and sequencing. Primer pair is designed on MethPrimer
(www. urogene.org/methprimer) F1 is the forward primer 5'-
TTTATTATTTATTGTGGTGGTGG, R1 is the reverse primer, 5'-
AATAAAAACCTTTACAAAAAAAACCCTAC, which cover the -561 to -266bp promoter region. Experiment was repeated once to confirm the results.

**G. Decitabine and Trichostatin A Treatment to Human Breast Cancer Cells**

MDA-MB-231, MDA-MB-468, MCF7, M4A4 cells were tested in this experiment. Decitabine is from Tocris (Cat. No. 2624), and trichostatin A (TSA) is from Sigma (T8552). On the day before experiment, plate 5x10^5 cells in 6-cm plates for individual treatment. For the experiment, DMSO, decitabine (5µM), TSA (300nM) or decitabine and TSA combination was treated to each cell for 72hr continuously. Three days later harvest total RNA and samples were applied to QPCR analysis. Samples were harvested from 3 independent plates for each treatment. Results were expressed as Mean ± SE.

**H. Western Blotting for HtrA1 in Human Breast Cell lines**

2µg of cytoplasmic and 12µg of nuclear portions for each cell were separated by 10% SDS-PAGE. Proteins were transferred to Immobilon-FL PVDF (Millipore, cat. # IPFL00010) membrane at 40V for 2 hr. Blots were blocked with 6% fat-free dry milk powder in TBS/0.1%Tween20 for 1.5 hr at RT, and then were incubated with primary antibody overnight at 4C. Using the HtrA1 monoclonal antibody from R&D systems (Cat. No. MAB2916, 1:500 dilution), we run the gel with non-reducing condition, which is recommended by the company. Secondary antibody was anti-mouse IgG, HRP-linked
(Cell Signaling Technology, #7076). As loading controls, β-actin mouse monoclonal antibody from Santa Cruz (#SC-47778) was used and for nuclear protein fractionation, we used a rabbit polyclonal anti-DEK antibody (Aviva Systems Biology, #P100637). This experiment was performed at least three independent times.

3. Results

A. HIC for Human Breast Tissues

With the HIC analysis, we observed that HtrA1 was highly expressed in normal ductal epithelium, but dramatically down-regulated in ductal carcinoma in situ (DCIS) and variably expressed in invasive cancers (Figure 3-1). However, interpretation must be processed with caution since the HtrA1 antibodies also show higher molecular weight bands on Western blots and the sample size of breast tissues also needs to be enlarged.
B. QPCR and Northern Blot Analysis

First, we looked at HtrA1 transcript levels by using quantitative real time RT-PCR in 7 breast cell lines including 5 carcinoma cells, MCF7, MDA-MB-231, MDA-MB-468, NM2C5, M4A4 and 2 immortalized non-tumorigenic cells, MCF10A and MCF12A. HtrA1 gene expression was dramatically decreased in breast carcinoma cells compared to non-tumorigenic counterparts. Changes are greater than 20 fold (P< 0.05 by

**Figure 3-1: Immunohistochemical staining of HtrA1 in human breast tissues.** Tissue sections were deparaffinized and hydrated before the immunohistochemical staining procedure. HtrA1 mouse monoclonal antibody (R&D Systems) was applied for primary incubation. The dark purple stains in normal breast ducts indicated a higher level of HtrA1 expression, while ductal carcinoma in situ (DCIS) and invasive tumors shown weak or absent staining for HtrA1. Results were presented as 20x and 40x magnitudes.
Student's paired t test), except for the MDA-MB-231 cell line, in which HtrA1 mRNA level is about half of that in the MCF10A cell line (Figure 3-2a). We further confirmed this expression difference with Northern blot analysis, using 1.5kb HtrA1 full-length coding region cDNA as the hybridization probe (Figure 3-2b).

C. gDNA-PCR and Coding Region Sequencing

It is possible that the reduced level of HtrA1 expression is due to chromosomal aberrations. Therefore, we first tested this hypothesis using HtrA1 gDNA PCR, which amplified the 500bp region covering the first exon of HtrA1 gene. We did not see the HtrA1 gene loss in any of the 5 breast cancer cell lines (Figure 3-3). To exclude the possibility of HtrA1 gene silencing in those cancer cells due to gene point mutations, we sequenced the 1.5kb coding regions for all 7 cell lines using mRNA RT-PCR (blasting with NCBI genbank reference NM_002775.3). Results indicated that there were no point mutations or deletions in all of the cell lines (data not shown).
Figure 3-2: HtrA1 gene expression in human breast cell lines. HtrA1 gene expression in breast cell lines was analyzed by RT-QPCR (a) and Northern blot analysis (b) with three independent biological samples. MCF10A and MCF12A are two immortalized non-tumorigenic cells and all the other five are breast cancer cells. Error bars represent the standard error of the mean. Northern blot analysis for HtrA1 gene expression in seven breast cell lines Blot was sequentially probed with HtrA1 1.5kb full-length cDNA probe and 2.1kb β-actin cDNA probe applied as an internal control. Expression levels were 20-25X higher in the non-tumorigenic MCF10A and 12A cell lines, with very low expression levels in most of the hBC cell lines (MDA-MB-231 was the exception).
Figure 3-3: HtrA1 gene copies in human breast cell lines. To examine the gene copies of HtrA1 in all seven breast cell lines, a semi-quantitative PCR was performed. 20ng gDNA for each cell line was used as the template for PCR reactions. The lower band for each cell on the gel is 500bp of HtrA1 gene, which covers its first exon. The upper band in each lane is 700bp of the single-copy gene for the Factor IX human blood clotting protein (access #K02402), as the gDNA loading control. Experiment was carried out with 24, 26, 28, and 30 cycles to avoid saturation of PCR amplification and we did not detect gene lost in any of the seven breast cell lines.
D. Bisulfite Sequencing Result

It is possible that decreased HtrA1 expression in breast cancer is attributable to epigenetic modifications, which are independent of the HtrA1 sequence.

Since the 800bp upstream of the HtrA1 gene transcription start site is GC expansive (including two CpG islands), epigenetic DNA hypermethylation was considered as an excellent candidate for investigation.

Using bisulfite gene sequencing, we observed an inverse correlation between mRNA levels and DNA methylation state from -561bp to -266bp of the HtrA1 promoter regions. Within this region, there are a total of 35 CpGs. In the MCF10A and MCF12A cell lines, 40.9% and 44.4% of those CpGs are methylated, while an average of 68.1% of MDA-MB-468 cell, 95.2% of NM2C5 cell, 98.4% of M4A4 cell and 100% of MCF7 cell are methylated within the same region. MDA-MB-231 cell did not show any methylated cytosines in the total 14 CpGs from the sequencing result (Figure 3-4A). The inability to define methylation status of many CpGs in the MDA-MB-231 cell line might be due to poor quality of the gDNA template after bisulfite conversion. However, the PCR product size of this sample was the same as other samples. We are currently determining the sequence of this CpG island in the MDA-MB-231 cell line.

Comparing HtrA1 mRNA expression results from QPCR and Northern analysis, it appears that its regulation involves the -501 to -415bp region of the promoter (Figure 3-4B). Among total 14 CpGs in this region, Cs in breast cancer cell lines were all methylated except in MDA-MB-231 cell, whereas the MCF10A and MCF12A cell lines have many unmethylated Cs.
Figure 3-4: DNA methylation of HtrA1 gene promoter. gDNA was extracted from the various cell lines and analyzed via bisulfite sequencing. Analyzed region is shown in the above chart, -561 to -266 bp (F1/R1 region) from the transcription start site. There are totally 35 CpGs within this region. The PCR sequencing results for each breast cell line is illustrated in panel A. Blue bars are representative for unmethylated cytosines. Red bars at each end of individual cell line divide the region that can be detected from sequencing results. In panel B, the potential gene expression association region of HtrA1 was further narrowed down to the -501 to -415bp of the promoter. Total 14 CpGs in this region were all methylated in breast cancer cells, MCF7, NM2C5, M4A4 and MDA-MB-468, whereas two normal cells, MCF10A and MCF12A, were having several unmethylated cytosines in this region. Cancer cell line MDA-MB-231 is an exception. All 14 cytosines were unmethylated in the same region. Experiment was repeated once to confirm.
E. Decitabine and/or TSA Treatment

To determine whether HtrA1 gene expression in human breast cancer cell lines is regulated by epigenetic modifications, we treated four breast cancer cell lines (MDA-MB-231, MDA-MB-468, MCF7, M4A4) with either DMSO (solvent control), decitabine (DNA methyltransferase inhibitor), TSA (histone deacetylase inhibitor) or decitbine and TSA in combination. Original MCF12A cell line was used as HtrA1 gene positive control for QPCR analysis. The results collectively indicated that HrA1 gene expression in MDA-MB-231 and MDA-MB-468 cells was inhibited by histone deacetylation, but not DNA hypermethylation whereas the gene silencing in MCF7 and M4A4 cells was largely due to DNA hypermethylation (Figure 3-5). In the MCF7 cell line, DNA methylation and histone deacetylation could be synonymous and coordinated to control HtrA1 gene expression. However, the restored HtrA1 gene expression levels in the MDA-MB-468, MCF7, and M4A4 cell lines were far less than the original HtrA1 expression level in the non-tumorigenic breast epithelial cell line MCF12A. Two possibilities could contribute to these results: the DNA methyltransferase and the histone deacetylase inhibition treatments were incomplete; some other mechanisms besides DNA methylation and histone deacetylation were involved. We further re-checked HtrA1 gene promoter region of the decitabine treated MCF7 sample by bisulfite sequencing. We found that all CpGs in the -501 to -415 region were demethylated after the treatment, while the original MCF7 cells showed them all to be methylated (Figure 3-4B). Data indicated that the DNA methyltransferase inhibition treatment was complete in this cell line. Therefore, either the histone deacetylase inhibition treatment was not complete, or besides DNA
methylation and histone modifications, the other mechanism, Chromatin remodeling, might also participate in regulation of HtrA1 gene expression in MCF7 cells.

Figure 3-5: Restoration of HtrA1 gene expression in human breast cancer cell lines. Breast cancer cells MDA-MB-231, MDA-MB-468, MCF7, M4A4 were treated with dissolvent control, DMSO; DNA methyltransferase inhibitor, decitabine; histone deacetylase inhibitor, trichostatin A (TSA); or the combination of decitabine and TSA. Total RNAs were then harvested and applied to QPCR analysis. Each sample came from three independent treated plates. The human normal breast cell line, MCF12A, was used as HtrA1 gene positive control for QPCR analysis. The y-axis represents normalized HtrA1 gene expression using DMSO treated MCF7 sample as 1. In the top panel, HtrA1 gene expressions in MDA-MB-231 and 468 cell lines are showing restored by histone modifications to different extent; but in MCF7 and M4A4 cells are not showing dramatic increase. Results for these two cell lines are enlarged to the lower panel. HtrA1 gene expression in M4A4 cell is increased by DNA demethylation, while MCF7 cell is showing slightly increase with both DNA and histone modification treatment. In order to confirm the DNA demethylation treatment was complete. The HtrA1 promoter in
decitabine treated samples was applied to the bisulfite sequencing. The potential HtrA1 expression association region -501 to -415bp of the promoter is showing all demethylation in the bottom panel. Representative data was shown from three independent experiments.

F. Immunoblotting for Human Breast Epithelial Cell Lines

We next checked HtrA1 protein levels in all seven human breast cell lines under non-reducing condition as recommended by the R&D Systems (Figure 3-6). HtrA1 showed as ~Mr 43,000-45,000, slightly higher than the Mr 35,000-38,000 doublet detected under a different condition, as well as some distinct higher Mr forms (which appear to represent doublets and triplets). Expression of HtrA1 protein was not observed in any of the five breast cancer cell lines, MCF7, NM2C5, M4A4, MDA-MB-231, and MDA-MB-468. This result indicates that there is post-transcriptional regulation of HtrA1 mRNA in the MDA-MB-231 cell line, since mRNA is observed in the absence of protein. HtrA1 expression was observed in the MCF12A and MCF10A/Flp cell lines, the latter of which was the parental cell line used to create HtrA1 overexpression or knockdown cells.
In summary, studies in this chapter provided evidence that expression of HtrA1 was dramatically reduced in all of the human breast cancer cell lines compared with the non-tumorigenic cell lines. There were no HtrA1 gene copy changes in those cancer cell lines showing in semi-quantitative PCR, and there were no gene mutations found in DNA sequencing analysis of cDNAs of all the breast epithelial cell lines. The down-regulation
of HtrA1 in breast cancer cells was associated with diverse epigenetic silencing mechanisms, including DNA methylation, histone deacetylation, or their combinations. And in the MDA-MB-231 cell line, HtrA1 expression also involved post-transcriptional regulation showing on the immunoblot.

It is increasingly being recognized that epigenetic changes play an important role in driving tumor initiation and progression. Because they are mitotically heritable, they could couple with genetic changes to cause such disease pathology. Three interconnected mechanisms, DNA methylation, histone modifications, and chromatin remodeling, are major causes for gene silencing in tumor genomes.

Besides the DNA hypermethylation, which was shown in our results, histone deacetylation is another possible reason for down-regulation of HtrA1 gene expression in human breast cancer cells. The direct supportive evidence came from Zupkovitz et al., who reported that mouse HtrA1 gene was one of those negatively regulated genes by mouse histone deacetylase 1 (HDAC1) [87]. By checking two regions of HtrA1 gene with chromatin immunoprecipitation (ChIP) assay, they found increased acetylation levels of histones H3 (AcH3) and H4 (AcH4) in HDAC1-deficient mouse ES cells compared to the wild-type cells. They also observed the loss of the repressive trimethylation features, trimethylated lysine9H3 (3MeK9H3) and trimethylated lysine27H3 (3MeK27H3), in the HDAC1-deficient cells.

These data support the idea that DNA methylation is closely linked to histone deacetylation, since class I deacetylases (including HDAC1) were found to be associated with both methyl-binding proteins and methyltransferases [224, 225].
Chromatin is composed of DNA wrapped around histone proteins. The fundamental structure of chromatin is the nucleosome. Nucleosomes are further compacted into higher-order structures with linker histones and nonhistone components [226]. DNA inside of such constructs is inaccessible to transcriptional machinery. Therefore, chromatin structure has to be dynamically modulated for gene expression.

Chromatin remodeling is executed by multi-subunit complexes, which include two basic groups of molecules, those which covalently modify histones or DNA, and the ATPase-dependent remodeling complexes [97]. The first group is composed of factors that can acetylate, methylate, phosphorylate, and ubiquitylate histones. They can be all involved in HtrA1 gene expression regulation.

In an attempt to look for crucial transcription factors for HtrA1 gene expression, we examined the proximal promoter region of human HtrA1 in TESS database, which provides potential transcription factors and their DNA binding sites for certain gene (http://www.cbil.upenn.edu). Data revealed numerous possible transcription factor-binding sites in the hypermethylated region (-501 to -415 bp) of HtrA1 promoter sequence, including T-Ag, NF-1, AP-1, Sp1, ER-α, c-Myc, c-Myb, E2F, and HOXA5. The chromatin structure of HtrA1 gene, and its interplay with DNA and histone modifications, might silence its expression by affecting the binding of those transcription factors to its promoter region.

Overall, the epigenetic silencing for HtrA1 gene expression could provide a possible strategy for re-activating HtrA1 gene expression in breast cancer cells, thus facilitating further investigation of HtrA1’s role in chemotherapy.
Chapter 4

Creation of HtrA1 Knockdown and Overexpression Cells in Parental MCF10A Cell

1. Introduction

The second goal for the thesis work was to study the potential functions of HtrA1 in breast carcinogenesis and chemoresistance. To this end, we originally planned to create stable cell lines for over-expressing HtrA1 in the MCF7 breast cancer cell line and knocking down and overexpressing HtrA1 in the non-tumorigenic MCF10A breast epithelial cell line.

To generate these stable cell lines, we used the Invitrogen Flp-In system for transfection of the pcDNA5 expression vectors containing either the full length HtrA1 1.5kb coding sequence, or shRNAs targeted to HtrA1. We totally have four different HtrA1 knockdown sites been selected for the transfections.

The siRNA pathway begins with cleavage of long double-stranded RNA by the Dicer enzyme complex. Then the siRNAs are incorporated into Argonaute 2(AGO2) and the RNAi-induced silencing complex (RISC). If the RNA duplex has perfect sequence complementarity, AGO2 cleaves the passenger strand so that active RISC contains the antisense strand. This strand recognizes target sites to direct mRNA cleavage carried out by the AGO2 [227].
In parallel, we tried to overexpress HtrA1 in breast cancer cell line MCF7. However, we were unable to obtain any clones overexpressing HtrA1 in the MCF7 cell line following hygromycin selection, even though control transfectants worked well, suggesting that re-expression of HtrA1 was lethal in MCF7 cells, potentially by increasing hygromycin-induced cytotoxicity. We will discuss more in later this chapter.

We next focused on the functional effects of HtrA1 expression using HtrA1 targeted siRNAs (HtrA1 knockdown cells) and HtrA1 overexpression cell line derived from the MCF10A cell line. We used the Flp-In system (Invitrogen) to create a stably transfected cell line containing a single recombination site. We then transfected this cell line with expression constructs containing either siRNAs targeting selected sites in the HtrA1 coding region, or containing the full-length HtrA1 coding region. The switch of β-galactosidase expression from on (pFRT/lacZeo in Flp-In host cell) to off (integration of expression construct) under hygromycin selection indicated positive clones containing the various constructs. Sequentially, immunoblot analysis confirmed the positive clones.

To study the consequences of HtrA1 down-regulation, soft agar assay was first performed, which tests for transformation of cells based on their ability to grow with anchorage independence.

In the last decade, the development of genomic analysis has considerably advanced modern biological research. Functional genomic analyses can now be performed on a global scale, examining DNA, RNA, and protein levels to provide tools for study of gene function and regulation, biomarker identification, disease classification, risk factor stratification and drug discovery [228]. Genomic analyses include gene expression array, microRNA array, comparative genome hybridization array, DNA
methylation array, genome-wide association studies (SNPs), proteomic analysis, somatic mutation analysis, and related bioinformatic and biostatistical analysis. To explore the potential unknown functions of HtrA1, we performed a human whole genome gene expression using the MCF10A-derived HtrA1 cell lines. Gene clustering analyses of the results indicated two major directions for further investigation on HtrA1’s potential role as a tumor suppressor: one involving the EMT and the other involving effects on the DNA damage response network (which are presented in Chapter 5 and Chapter 6, respectively).

2. Materials and Methods

A. Generation of HtrA1 Overexpression or Knockdown Stable Cell Lines

Invitrogen Flp-In system was used to create stable expression cell lines. The strategy of this system transfection is illustrated in Figure 4-1. The major components of this system include: a Flp-In target site vector, pFRT/lacZeo, for generation of a host cell line containing an integrated FRT site; an expression plasmid containing a FRT site linked to the hygromycin resistance gene for Flp recombinase-mediated integration and selection of a stable cell line expressing the gene of interest under the control of the human cytomegalovirus (CMV) immediate-early enhancer/promoter; and a Flp recombinase expression plasmid, pOG44, for expression of the Flp recombinase under the control of the human CMV promoter.
The experiment procedure included two-step transfections. First, pFRT/lacZeo plasmid DNA was purified and linearized with ApaI and then transfected into the parental MCF10A cell line. Zeocin antibiotic (80 µg/ml) was used for selection of stable integrants. MCF10A/Flp positive clones were verified by β-gal staining and screened by Southern analysis to identify one-copy clones for the next step of transfection. Second, the pcDNA5/FRT expression vectors containing either HtrA1 full-length coding sequence, which was driven by human CMV promoter, or HtrA1 targeted siRNAs, which were driven by two opposing PolIII promoters H1 and U6, were co-transfected with pOG44, a plasmid encoding the Flp recombinase, into MCF10A/Flp cell line. Hygromycin antibiotic (30-40 µg/ml) was used to select cells stably overexpressing HtrA1 or stably expressing the HtrA1-targeted siRNAs. Positive clones were checked for HtrA1 gene expression levels and the loss of β-gal activity.

The full-length mRNA of HtrA1 is 2133 bp (NM_002775 in NCBI GenBank), and its full-length coding sequence is 1443 bp long (113-1555 bp). Those four HtrA1 siRNAs have been selected by our library selection method, which were showing effective down-regulation of HtrA1 mRNAs in vitro by transient transfection experiments. The siRNAs sequences are: siRNA1, 5’-GCCGCGGTCATCGTCTG (544-564 bp); siRNA2, 5’-CCGTGGTTCATATCGAATTGT (659-679 bp); siRNA3, 5’-GGTGCCACTTACGAAGCCAAA (819-839 bp); and siRNA4, 5’-CACGGAGTCCCATGACCAGCA (1204-1224 bp).
Figure 4-1: Diagram of the Flp-In System from Invitrogen. This figure illustrates the major features of the Flp-In system as described on the left.
Figure 4-2: Subcloning for creation of HtrA1 overexpression and knockdown constructs. On the left, HtrA1 overexpression plasmid was created by cutting out 1.5kb coding sequence from TOPO vector, inserting it into pcDNA5/FRT construct. On the right, each of the HtrA1 21nt antisenseoligos was in the OPIII cassette in the pSilencer construct. OPIII cassette is composed of two strong opposing PolIII promoters, H1 and U6. By similar way, each antisenseoligo within in the OPIII cassette was cut out from the pSilencer construct and inserted into pcDNA5/FRT vector. After transcribed in cells, HtrA1 siRNAs were produced.

Figure 4-3: Diagram for HtrA1 siRNA targeting positions. On HtrA1 2133bp full length mRNA, siRNA1 targets the sequence, which will otherwise encode Kazal protease inhibitor (KI) domain; siRNA2, 3, 4 target the sequences encoding the trypsin-like protease domain.
B. β-gal Staining for Stable Cells

3-4x10^5 cells for each cell line were plated in a 6-well plate the day before experiment. On the next day, cells were washed with 2ml PBS/ 2mM MgCl₂ and then were fixed with 1ml fix solution (37% formaldehyde 50µl/ml, 25% glutaraldehyde 2µl/ml, in PBS) at RT for 5min. Cells were washed twice with PBS, and then substrate/staining solution was added (50mM K⁺-ferricyanide 0.1ml/ml, 50mM K⁺-ferrocyanide 0.1ml/ml, 1M MgCl₂ 2µl/ml, and 1mg/ml X-gal in PBS) and incubation was for 2hr to overnight at 37°C. Cells were examined and photographed using an inverted microscope.

C. Immunoblotting for HtrA1 in Flp-In Transfected Cell Lines

Cell culture medium was collected after plating cells for 48hr and concentrated using Microcon YM-3 centrifugal filter devices (Cat. # 42404) by spinning at 13,000 rpm at 4°C, 30 min for 3 times. Cells were extracted with NE-PER nuclear and cytoplasmic extraction reagents (Thermo Scientific #78833). 2µg of cytoplasmic, 12µg of nuclear, and 180µg medium protein were separated by 10% SDS-PAGE. Proteins were transferred to Immobilon-FL PVDF (Millipore, cat. # IPFL00010) membrane at 40V for 2 hr. The blot was blocked with 6% fat-free dry milk powder in TBS/0.1% Tween20 for 1.5hr at RT. Primary antibody, rabbit polyclonal anti-human HtrA1 (Imgenex, IMX-6518A), was added at 1:5000 dilution, and the blot was incubated overnight at 4°C for the cytoplasmic and nuclear fractions, while the mouse monoclonal anti-human HtrA1/Prss11 (R&D Systems, Cat. No. MAB2916) was used 1:500 dilution for the medium portion.
Secondary antibodies were anti-rabbit IgG, HRP-linked (Cell Signaling Technology, #7074) and anti-mouse IgG, HRP-linked (Cell Signaling Technology, #7076). As loading controls, β-actin mouse monoclonal antibody from Santa Cruz (#SC-47778) was used and for nuclear protein fractionation, we used a rabbit polyclonal anti-DEK antibody (Aviva Systems Biology, #P100637). This experiment was performed at least three independent times.

**D. Soft Agar Assay for Colony Formation**

Prior to the experiment, 3 reagents were prepared. 1. 2x DMEM/F12 medium + 10% heat-inactivated horse serum; 2. 1% base agarose layer and 0.6% top agarose solution with Seakem GTG agarose (Cambrex Bio Science Rockland, Inc. Cat. No. 50071); and 3. 60mm petri dishes for cell culture (Falcon #35-1007). On the beginning day, a 1:1 mix of 1% base agar and 2x medium was prepared and 3ml was added to each plate. This was allowed to solidify. Harvested cells were counted (in 2x medium) and 7500 cells in 1.5ml of 2x medium was added to each plate. A 1:1 mix with 0.6% top agar and 2x medium was made, and 3ml was added on top of the base agar layer. Plated were incubated for 4 weeks, with the cells fed twice a week with fresh 2x medium (1.5ml). For counting colonies, plates were stained with 1ml 0.006% Crystal Violet (Sigma-Aldrich, HT901) for 3hr. Colonies were counted on 3 plates for each cell line at each time point examined.

**E. Microarray Analysis**
Microarray analysis was performed using the Illumina Human Whole Genome Beadchip assay (Illumina, San Diego, CA) in the PSU-COM Functional Genomics Sciences Core Facility. RNA quality and concentration was initially assessed using an Agilent 2100 Bioanalyzer and an RNA Nano LabChip (Agilent, Santa Clara, CA). cRNA was synthesized by TotalPrep Amplification (Ambion, Austin, TX) from 500 ng of starting RNA according to manufacturer’s instructions. T7 oligo (dT)-primed reverse transcription was used to produce first strand cDNA. cDNA then underwent second strand synthesis and RNA degradation by DNA Polymerase and RNase H (respectively), followed by a filtration clean up. In vitro transcription (IVT) was employed to generate multiple copies of biotinylated cRNA. The labeled cRNA was purified using filtration, quantified by NanoDrop, and volume-adjusted for a total of 1.5 ug/sample. Samples were fragmented, and denatured before hybridization for 18 hours at 58°C. Following hybridization, beadchips were washed and fluorescently labeled. Beadchips were scanned with a BeadArray Reader (Illumina, San Diego, CA).

A project was created with resultant scan data imported into GenomeStudio 1.0 (Illumina). Results were exported to GeneSpring 7.3 (Agilent Technologies). Measurements less than 0.01 were then set to 0.01, arrays normalized to the 50th percentile, and individual genes normalized to the median of controls.

Silhouette measurement was used to determine the optimal number of clusters.

To determine the optimal number of clusters we used the Silhouette width index (Kaufman and Rousseeuw, 1990). This Silhouette width for each point is a measure of how close that point is to the points of its own cluster compared to points in other clusters: $s(i) = b(i) - a(i) / \max(a(i),b(i))$ with $b(i) = \mink(B(i,k))$ where,
s(i) = Silhouette width for each point i

a(i) = The average distance between point i and points of its own cluster

B(i,k) = The average distance between point i and points of another cluster k

b(i) = The minimum of the average distance between point i and all other points in other clusters

The Silhouette width value ranges from -1 to 1.

- A value close to 1 means that the sample is well clustered.
- A value close to zero means that it could be assigned to another cluster as well.
- A value close to -1 means that the point i has been most likely misclassified.

We can compute the average Silhouette width for each cluster and for entire dataset. The optimal number of clusters would be the one that maximizes the overall Silhouette width for the entire dataset.

3. Results

A. β-gal Staining to Verify the Stable Transfection Cell Lines

The MCF10A Flp-in host cell line was created by transfection of the MCF10A cell line with a construct containing a recombination site, which was selected for lacZ-Zeocin expression. Positive clones therefore exhibited β-galactosidase activity after selection using Zeocin. After the second transfection with the HtrA1-targeted siRNA
constructs (or the HtrA1 expression construct), the constructs were integrated into recombination site in the host cell line, which converted them to Zeocin sensitivity (via loss of β-galactosidase activity) under Hygromycin selection. X-gal staining for all the HtrA1 transfected cells was used to verify the positive clones (Figure 4-4).

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**Figure 4-4: Verification of the stable transfected clones with β-galactosidase activity.** Original MCF10A cell does not have β-galactosidase activity. MCF10A/Flp, the intermediate host cell line for creating HtrA1 transfected cells, express β-galactosidase under Zeocin selection. After the second step of transfection, HtrA1 cell lines (overexpression or knockdown) were rendered Zeocin sensitive (due to lack of β-galactosidase activity) and acquired Hygromycin resistance, allowing them selection using Hygromycin.
B. Western Blotting for Detection of HtrA1 Protein Levels

Expression of HtrA1 in the overexpressing cell line, designated MCF10A/HtrA1, and in knockdown cells, designated MCF10A/siRNA1 through MCF10A/siRNA4, was evaluated by Western blot analysis. A rabbit polyclonal antibody against a 22aa region within the HtrA1 protease domain was used for detection. This antibody detected a doublet of bands both at ~Mr 35-38,000 in the cytoplasmic extracts. In the nuclear fractions, in addition to the doublet bands, it also detected a proteolytic form of HtrA1 ~Mr 29,000. However, this HtrA1 polyclonal antibody did not detect any bands from cell culture media. By using the HtrA1 mouse monoclonal antibody (R&D Systems), the secreted HtrA1 was detected as a band ~Mr 40,000 in the cell culture media. We observed an increase of HtrA1 expression level in overexpressing MCF10A/HtrA1 cell line and decreased expression in the knockdown MCF10A/siRNA cell lines in both cytoplasmic and nuclear fractions (Figure 4-5). We did not observe different levels of HtrA1 in the medium comparison. Subsequent experiments using medium blanks also showed no staining for HtrA1 (not shown).
C. Soft Agar Assay for Testing Tumorigeneity of HtrA1 Down-regulated Cells

In soft agar assays conducted for 5 weeks, there were no colonies detected in MCF10A-siRNA knockdown cells (siRNA1/2/3/4), nor in the parental MCF10A cell line, the MCF10A-Vector control cell line or with the MCF10A-HtrA1 overexpressing...
cell line. The MCF7 cell line, which is a transformed breast cancer cell, was used as a positive experimental control for growth of anchorage independent colonies. The MCF7 colonies started to form ~5 days after plating, growing gradually bigger and reaching maximal size at the 28th day, after which the colonies gradually shrunk. Although deduction of HtrA1 expression did not result in obvious transformation of the MCF10A cell line, we suggest that loss of HtrA1 expression may represent a very early step in transformation, and might increase risks of subsequent genetic and epigenetic alterations, which could subsequently result in cellular transformation.

Figure 4-6: Soft agar assay for detection of tumorigenesis in HtrA1 knockdown cells. Representative data are shown from multiple pictures taken on the same day, the 28th day after plating cells. Human breast cancer cell MCF7 is the positive control for colony growth. The various MCF10A-derived cell lines did not exhibit transformed colonies throughout the entire 35-day experiments. Colonies were counted on 3 plates for each cell line at each time point examined.
D. Gene Array Analysis

To compare gene expression profiles with altered HtrA1 gene expression levels in the series of MCF10A-derived cell lines, we used the HtrA1 overexpression cell 10A/HtrA1, two HtrA1 knockdown cell lines, MCF10A/siRNA3 and MCF10A/siRNA4, and a cell line expressing an siRNA control cell, MCF 10A/HPVsh, with the Illumina Human Whole Genome Beadchip (Illumina, San Diego, CA). We used two methods to analyze data, including gene cluster analysis and by signaling pathways. First, by considering the genes whose fold change in expression levels is greater than 50%, 1402 genes were identified for clustering analysis. We used the Silhouette measurement to determine the optimal number of clusters (Figure 4-7A). Using this analysis, the optimal number of clusters was determined to be 22 (Silhouette width = 0.3841). We were interested in both genes whose expression was inversely correlated with HtrA1 expression level, and genes whose expression was positively correlated with HtrA1 expression levels in the cell lines.

Cluster 19, 6, and 2 were the top three clusters (based on the magnitude of change in gene expression) whose expression was inversely correlated with HtrA1 expression level (Figure 4-7B). Cluster 12, 21, 4, and 11 were the top four clusters whose expression levels were positively associated with HtrA1 expression level (Figure 4-7C illustrates representative genes in each of these top clusters).

Results suggested that at least four functional clusters of genes were changed correlated to HtrA1 gene’s level. First, many EMT marker genes and EMT-related genes were significantly changed when HtrA1 was down-regulated. Genes showing increased
expression included vimentin (VIM), PTPLA, PTPRE, HIF1A, FGFR3, VEGFB, and VEGFC. Genes showing significantly decreased expression included E-Cadherin (CDH1), claudin1 (CLDN1) and claudin7 (CLDN7), and cytokeratins (KRT13, 15, 16). A second group of genes whose expression was up-regulated in the MCF10A/siRNAs cell lines included many components of the ATM initiated DNA damage response network, such as, ATM, and its substrate H2AFX, as well as RAD21 et al. Thirdly, basic histones (such as HIST1H1C, HIST1H2AC, HIST2H2AA3) showed expression which was positively correlated with HtrA1 expression level, so that HtrA1 could be involved in gene transcription regulation by effects at the level of nuclear histones. Further, we found several genes that might relate to cell migration, showed increased expression when HtrA1 expression was down-regulated, including TNFRSF6B (the tumor necrosis factor), MTA1, LAMB1, and et al.
Figure 4-7A: Clustering Analysis for the Whole Genome Gene Array. Summary of the gene clusters identified in MCF10A-derived cell lines. Gene expression levels in HtrA1 overexpression and knockdown cells were normalized with vector control cell. By considering the genes whose fold change in expression levels is greater than 50%, 1402 genes were identified for clustering analysis. Totally 22 clusters were determined by the silhouette measurement.
Figure 4-7B: Genes inversely correlated with HtrA1 expression. Cluster 19, 6, and 2 are the top three clusters based on gene expression changing fold(s). Complete gene set was shown for Cluster 19 and 6, respectively. Sample genes were shown in cluster 2.
**Genes positively correlated with HtrA1 expression**

********** CLUSTER 12 **********
The total number of genes in this cluster = 10
CD24, C1ORF116, KLK7, CD24, KLK5, SPRR2D, KRT13, KLK5, KLK10, MFAP5

********** CLUSTER 21 **********
The total number of genes in this cluster = 23
MUC1, THBS1, SCD5, COL8A1, HIST1H2BD, HIST1H1C, RARRES1, PTGS2,  
HIST1H2AC, FAP, RARRES1, SPRR2F, SERPINB2, SERPINB2, SERPINB7,  
LOC728285, NLRP3, VGL1, A2ML1, PHACTR3, KRT34, CSAG1, SPRR2A

********** CLUSTER 4 **********
The total number of genes in this cluster = 22
TACSTD2, KRT15, RNASE7, SLC4A2, CMTM7, CLDN7, FOXQ1, OVOL2,  
TAGLN, CDH1, IL24, TMEM125, CST6, CST6, LOC642299, PROM2, PR558,  
LOC647859, FXYD3, IL24, FXYD3, FXYD3

********** CLUSTER 11 **********
The total number of genes in this cluster = 35
MAL2, LOC650517, LOC650428, KRT16, RG52, PLAUR, CDKN1A, CLDN1, FST,  
ADM, ADRB2, F3, HIST2H2AA3, MGC102966, HIST2H2AA3, DMBT1, PLAUR,  
LOC400578, KRT16, C1ORF116, PXDN, KLK6, C8orf13, PLAUR, GPRC5A, PLD5,  
LOC729252, C1IC3, CRABP2, MGLL, KLK5, MUC16, LOC400578, IGFL1, KLK8

**Figure 4-7C:** Genes positively correlated with HtrA1 expression. Cluster 12, 21, 4, and 11 are the top four clusters in this category. Complete gene set was shown in each cluster.
4. Discussion

In attention to create the HtrA1 overexpression MCF7 stable cell line, we performed more than three independent times of transfection. We tried multiple transfection reagents as well as varied selection conditions, but we were unable to produce an MCF7-derived cells overexpressing HtrA1. These results suggest that overexpression of HtrA1 in this breast cancer cell line could induce apoptosis, potentially via increasing Hygromycin-induced cytotoxicity, which could support the tumor suppressor role of HtrA1. However, to test this hypothesis, we will need a model that we can monitor the apoptosis inducing process. We propose to create a model of inducible expression of HtrA1 in MCF7 and other breast cancer cell lines by using constructs such as Clontech’s Retroviral Tet-On Advanced or Retroviral Tet-Off Advanced Inducible Expression Systems. In this way, we should be able to monitor the inducible effects of HtrA1 re-expression on cellular phenotype.

In our immunoblot result for the various transfected MCF10A cell lines, we detected an Mr 29,000 band in the nuclear fraction in addition to the Mr 35-38,000 doublet bands in the literature. We have shown this band to be proteolytically active [229]. A recent report from Lorenzi et al. (2008) also reported a significant up-regulation of an ~Mr 30,000 HtrA1 form in pre-eclampsia by quantitative western blotting analysis [230]. Thus, what was originally reported as an active nuclear form of HtrA1 may also be active elsewhere.
Although HtrA3 shows high homology with HtrA1 in amino acid sequence [27] and has similar expression patterns with HtrA1 in normal and cancer tissues of human endometrium [231], we did not detect HtrA3 expression in immortalized normal breast cell line MCF10A by QPCR analysis, in spite of finding high levels of HtrA1 expressions. This excluded the possibility that the observed HtrA1 knockdown phenotypes may reflect down-regulation of HtrA3.

Genomic studies usually generate a large amount of data, which need to be analyzed. Even after statistical processing, we may still observe a large number of dys-regulated genes. Bioinformatic tools can help to extract useful data to explain potential function ramification. One of the most commonly used tools is Gene Ontology (GO, http://www.geneontology.org/). Another popular analysis tool is Gene Module Analysis [232]. It uses known functions to categorize the gene array results, for example, by motifs for transcription factors, downstream substrates within one signaling pathway, etc. Another popular tool is Gene Set Enrichment Analysis (GSEA), which is publically available (http://www.broad.mit.edu/gsea/). We used the Silhouette width index (Kaufman and Rousseeuw, 1990) to determine the optimal number of gene clusters (based on gene expression changing fold) of our microarray results. By using this method, we concentrated on the most significantly changed genes in groups. And our analysis result suggested that HtrA1 gene might have two major functions as a potential tumor suppressor. One appears to be preventing the EMT, and another aspect may involve important effects on the DNA damage response network. Additional functional studies for these two directions will be presented in Chapters 5 and 6.
Chapter 5

Down-regulation of HtrA1 Renders Cells an EMT Phenotype

1. Introduction

From the microarray results, we get the information of that down-regulation of HtrA1 might be involved in the EMT.

During normal embryonic development, in the formation of early stage of metazoan, cells from epithelium are internalised to produce the mesodermal tissue. Therefore those cells need to detach from junctions connected to the neighbour cells, change their shape and polarity, and migrate. This morphological event is called the EMT. During gastrulation and formation of organs like the neural crest, heart, muscular system, craniofacial structures and peripheral nervous system, the EMT is required [108].

Parallels exist between tumorigenesis and the process of wound healing during tissue injury, both involving the recruitment of mesenchymal cells and then differentiation into various epithelial lineages through mesenchymal to epithelial transitions [233]. An essential difference between the embryonic and tumorigenic processes is the latter involve genetically abnormal cells, which evolve with gradual loss of responses to normal growth-regulatory signals.

Recently, the EMT, as well as cancer stem cells, have been suggested to be mechanisms which could facilitate chemoresistance [121, 160-162]. For example, higher E-cadherin expression in cells is related to higher sensitivity to EGFR kinase inhibitors,
while mesenchymal-like cells are more drug-resistant [234]. Cancer cells can also undergo adaptive changes after therapy to develop drug resistance that may involve the programs like the EMT [171].

As stated, HtrA1 has been found to be one of a trio of genes (PRSS11, MTSS1, CLPTM1) could distinguish doxorubicin-responsive from non-responsive breast tumors in 95% of samples [10], and we have shown in chapter 3 that HtrA1 was dramatically down-regulated in breast cancer cells compared with normal cells.

The key events in the EMT during tumor progression include down-regulation of E-cadherin, and acquisition of a highly motile and invasive phenotype, with overexpression of Vimentin and other mesenchymal markers. Recently, a series of novel EMT-inducing transcription factors was identified, including HOXB7, which is a member of homeobox family [235], the forkhead transcription factor FOXC2 [236] and the fibroblast-specific protein 1-inducing transcription factor, CBF-A [237]. These are in addition to the “classic” EMT-inducers, such as zinc finger transcription factors, ZEB1 (δEF1), ZEB2 (SIP1) and bHLH factors E12/E47 (TCF3) and Twist. Loss of cell-cell adhesion and apical-basal polarity, as well as the acquisition of fibroblastoid motility is another typical phenotype of EMT.

Our microarray results show significant changes for almost all of the above-mentioned molecules. To examine elements of EMT phenotype in HtrA1 knockdown cells, we first verified the changes in transcript expression of VIM and E-cad by QPCR analysis. Then we looked at the epithelial cell markers, E-cad and KRT5/6/18, and mesenchymal markers, VIM and N-Cad, by ICC. Next, we tested cell migration and invasion ability on transwell plates.
To date, many different extracellular cues have been shown to trigger epithelial
de-differentiation and the EMT, such as those involving TGFβ, Notch, FGF, and Wnt
signaling pathways [109], microRNAs, HIFα- and NFκB-dependent mechanisms, as well
as steroid hormone signaling [102].

We performed microRNA array analyses to investigate the effects of decreased
HtrA1 expression level in MCF10A-derived cell lines. We also examined the activation
of EGFR and AKT by Western Blotting analysis. Further, we examined the regulation by
TGFβ1 in HtrA1 knockdown-rendered EMT phenotype.

Finally, to test the role of HtrA1 in chemosensitivity of breast cells, we treated
two groups of cells with the drug of doxorubicin. The first group included cancer and
normal breast cells used in Chapter 3 for HtrA1 gene expression characterization, and the
second group included the various MCF10A-derived cell lines HtrA1 (MCF10A/siRNAx,
etc).

### 2. Materials and Methods

#### A. Cell Growth Rate

MCF10A/HPVsh, MCF10A/HtrA1, MCF10A/siRNA1, and MCF10A/siRNA4
cell sizes were manually measured using ImageJ program ([http://imagej.nih.gov/ij](http://imagej.nih.gov/ij)). 50
cells for each cell line were randomly chosen for cell area counting.

To monitor the cell growth rate in the MCF10A/siRNA and MCF10A/HtrA1
transfected cell lines, 5x10^4 cells for each cell line were plated in 10cm plates on day 0.
Cells were continuously cultured for 6 days in DMEM/F12 medium + 5% Horse serum, 20 ng/ml human epidermal growth factor, 0.01 mg/ml bovine insulin, 100 ng/ml cholera toxin, 500 ng/ml hydrocortisone, 95% and 30 µg/ml Hygromycin selection antibiotic. Cells were counted on day 2, day 4 and day 6, with triplicate plates for each count. Results were expressed as means ± SE. Student’s paired t test was used to determine statistical significance (Microsoft Excel). Values were considered as statistically significant if P<0.05.

B. QPCR Verification for CDH1 and VIM Gene Expression

Total RNAs from MCF10A, MCF10A/HPVsh, MCF10A/HtrA1, MCF10A/siRNA1, and MCF10A/siRNA4 cells were extracted with Qiagen RNeasy mini kit (cat. #74904) in three independent experiments. Quantitative real-time PCR was performed with the QuantiTect Probe RT-PCR kit (Qiagen, Cat. # 204443) on a Stratagene Mx4000 QPCR system (Agilent Technologies). All quantification data were normalized to TBP, which acts as an internal control. The following primers and probes were used for human genes: vimentin, 5’-AGATGGCCCTTGACATTTGAG-3’ (forward), 5’-CCAGAGGGAGTGAATCCAGA-3’ (reverse), 5’-FAM-ACCTACAGGAAGCTGCTGGAAGGCCG-BHQ-3’ (probe); for human CDH1, 5’-CGACCCAAACCCAGAATCTA-3’ (forward), 5’-AGGCTGTGCCTTCTCTACAGA-3’ (reverse), 5’-FAM-AGCAGGACTGATTGCTACGAGGTGGGT-BHQ-3’ (probe); for human TBP, 5’-CACGGGACTGATTGCTACGAGGTGGGT-BHQ-3’ (probe); for human TBP, 5’-CACGGGACTGATTGCTACGAGGTGGGT-BHQ-3’ (probe).
Results were expressed as means ± SE. Values were considered as statistically significant if P<0.05 (Student’s paired t test).

C. Immunofluorescence Cytochemistry

Cells were plated at 2-3x10⁴ cells/cm² on chamber slides the day before staining. On the experiment day, cells were rinsed with PBS, then fixed in 10% neutral buffered formalin for 20 min at RT. After rinsing with PBS, cells were permeabilized in PBS/0.2% Triton-X for 10 min at RT, blocked with 2.5% secondary species serum in PBS/0.1% triton-X for 1 hr at RT. Primary antibody was diluted in the same blocking buffer and incubated with samples at 4°C overnight. On the following day, cells were washed with PBS/0.2% Triton-X 3X3 min, and incubated with secondary antibody for 1 hr at RT, and washed with PBS/0.2% Triton-X 3 times for 3 min each. After the final wash, DAPI was used to counterstain cells for 5 min, and slides were mounted and examined. Experiments were performed more than three independent times.

Antibodies used for ICC were:

1. Mouse monoclonal to Vimentin (V9) (NeoMarkers)
2. Donkey anti-mouse secondary antibody (Cy3)
3. Mouse monoclonal to Cytokeratin 5+6+18 (ab49289)
4. Rabbit polyclonal secondary antibody to mouse IgG-H&L (FITC) (ab97045)
5. Rabbit polyclonal to N Cadherin (ab12221)
6. Goat polyclonal secondary antibody to Rabbit IgG-H&L (Biotin) (ab6720)
7. Streptavidin-TRITO (Invitrogen #43-4314)
8. Rat monoclonal to E cadherin (ab11512)
9. Rabbit polyclonal secondary antibody to Rat IgG-H&L (FITC) (ab6730)
D. Cell Migration and Invasion Assay

96 well HTS transwell permeable supports with 8µm pores from Corning (Cat. No. 3374) was used for migration/invasion assays. 5x Basement Membrane Extract (BME) coating solution was obtained from Trevigen (Cat. No. 3455-096-02). Cell migration/invasion ability was quantified by Calcein-AM (Biotium #80011-3) detected with a Synergy HT plate reader with KC4 software (Bio-Tek Instruments, Inc).

Day1. Cells were starved and transwell inserts were coated with 1x Basement Membrane Extract at 37°C, in 5% CO2, overnight. Some of the wells were left uncoated as migration comparisons.

Day2. 4x10^4 cells were plated in each transwell insert, and stimulated with FBS attractant. A few wells without cells were included for background blank.

Day3. A standard curve was set up and cells that pass through the membrane were detected with fluorescent plate reader (485nm excitation, and 520nm emission filters).

The standard curve for this assay is the formula to calculate how many cells passed through the transwell based on the fluorescence value for each individual cell line.

Results were expressed as means ± SE. Values were considered as statistically significant if P<0.05 (Student’s paired t test).

E. MicroRNA Array Analysis

Experiments were performed using the Illumina v2 Human MicroRNA Assay Beadchip (Illumina, San Diego, CA) in the PSU-COM Functional Genomics Core Facility. RNA quality and concentration was assessed using an Agilent 2100 Bioanalyzer
with RNA Nano LabChip (Agilent, Santa Clara, CA). Then cRNA was synthesized from 200 ng of total RNA according to manufacturer’s instructions. The method targets specific sequences with sets of oligonucleotides, which are extended, and labeled during PCR amplification. MiRNAs were polyadenylated using Poly-A Polymerase (PAS, Illumina). The introduced poly-A tail was then used as a priming site for cDNA synthesis. The primer used in cDNA synthesis was biotinylated and contained a universal PCR primer sequence. The biotinylated cDNA is annealed to miRNA-specific oligonucleotides that correspond to all of the targeted microRNAs (1146 human microRNAs). The resulting single-stranded fluor-labeled PCR product was hybridized on the beadchip overnight with a temperature ramp from 60°C to 45°C. Following hybridization, beadchips were washed and scanned with a BeadArray Reader (Illumina, San Diego, CA). A project was created with resultant scan data imported into GenomeStudio 1.0 (Illumina). Results were exported to GeneSpring 7.3 (Agilent Technologies). Measurements less than 0.01 were then set to 0.01, arrays normalized to the 50th percentile, and individual genes normalized to the median of controls.

**F. Western Blot Analysis for EGFR/AKT Pathway**

Cells were extracted with NE-PER nuclear and cytoplasmic extraction reagents (Thermo Scientific #78833). 8 µg for each extraction portion was separated by 10% SDS-PAGE electrophoresis. Proteins were transferred to Immobilon-FL PVDF (Millipore, cat. # IPFL00010) membrane at 40V for 2 hr. The blot was blocked with 4% fat-free dry milk powder in TBS/0.1% Tween20 for 1 hr at RT. Primary antibodies were incubated overnight at 4°C. They were rabbit polyclonal anti-EGFR (Cell Signaling Technology,
(#2232), rabbit monoclonal anti-phospho-EGFR (Tyr998) (Cell Signaling Technology, #2641), rabbit polyclonal anti-Akt (Cell Signaling Technology, #9272), rabbit polyclonal anti-phospho-Akt (Ser473) (Cell Signaling Technology, #2232). Secondary antibody was anti-rabbit IgG, HRP-linked (Cell Signaling Technology, #7074). As loading control, β-actin mouse monoclonal antibody from Santa Cruz (#SC-47778) was used, and for nuclear fraction, we used rabbit polyclonal anti-DEK antibody (Aviva Systems Biology, #P100637). Experiment was performed at least three independent times.

G. Elisa Analysis for TGFβ1

Detection of human TGFβ1 was performed with the various HtrA1 overexpressing and knockdown MCF10A-derived cell lines using Quantikine Human TGFβ1 Immunoassay kit is from R&D systems, Inc. (cat # DB100B). Briefly, 20 µl 1N HCl was added to 100µl cell culture medium supernates, and incubation was at RT for 10 min to activate the samples; then were neutralized by adding 22µl 1.2N NaOH/0.5M HEPES. 258µl “calibrator” was added to give a 1:4 dilution for each sample. 50µl of activated standard, control medium, and samples were added to each well; then were incubated for 2 hr at RT. After washing, add 100µl of TGFβ1 conjugate was added to each well, and incubation was continued for another 2 hr at RT. After washing, 100µl of substrate solution was added, and incubation was for 30 min at RT in the dark, followed by addition of 100µl stop solution. The optical density of each well was determined by using microplate reader at 450nm, with wavelength correction at 540nm.
**H. Doxorubicin Cytotoxicity Test on Human Breast Cells**

1000 cells/well for each cell line were plated in 96-well plates the day before the experiment. On the experiment day, a series of concentrations of Doxorubicin HCl (from APP pharmaceuticals, LLC. NDC 63323-883-05) were added to each cell line for 48hr continuously (0, 0.002, 0.01, 0.05, and 0.25 µg/ml). Sample size was seven for each concentration tested. Surviving cells were counted two days later, using cell counting kit-8 (CCK-8) from Dojindo Molecular Technologies, INC. Briefly, 10 µl of CCK-8 solution was added to each well of the plate, and the plate was incubated for 3hr in an incubator, and the absorbance was measured at 450nm using a microplate reader. Experiment was repeated once to confirm the tendency.

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**3. Results**

**A. Cell Morphology and Growth Rate**

Compared to the control MCF10A/HPVsh cells, the HtrA1 knockdown cells were more sparse and smaller but appeared to grow faster, while the MCF10A/HtrA1 overexpressing cells were larger but appeared to grow slower. To verify these observations, we first measured cell sizes for those transfection cell lines and we found that compared to the control cell, down-regulation of HtrA1 (MCF10A/siRNA1 and MCF10A/siRNA4) decreased cell area for about 40%, whereas overexpressing HtrA1
MCF10A/HtrA1 increased cell area for about 1.5 fold, although it had a larger variation for this cell line (all three p-values < $1 \times 10^{-8}$) (Figure 5-1).

Simultaneously, cell growth experiments were performed for 6 days. We found that down-regulation of HtrA1 significantly increased cell growth rate, while overexpression of HtrA1 induced a slightly slower growth. Compared to the irrelevant control cell, both HtrA1 knockdown clones grew faster with p-values < 0.01 at both day 4 and day 6 (Figure 5-1).
B. Gene Array Summary for EMT and QPCR Verification

Our second method to analyze the microarray data was to summarize expression changes by signaling pathways. Specific in this chapter, genes (p-value < 0.05 by student’s t-test) involved in the EMT were summarized.

The first group is of EMT marker genes. As mentioned in the previous chapter, down-regulation of HtrA1 significantly decreased epithelial genes, CDH1, CLDNs, and quite a few KRTs, whereas HtrA1 exogenous expression increased those molecules to some extent. On the contrary, vimentin, ECM2, and LAMB1, etc., which are mesenchymal marker genes, were dramatically increased in HtrA1 knockdown cells (Table 5-1).

Direct regulators of these genes include several classes of transcription factors. We found that the bHLH factors such as E12/E47 (TCF3) and Twist, and recently identified forkhead family members, homeobox family genes, as well as a few of other

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**Figure 5-1: Morphological and growth rate changes in transfected MCF10A cells.** Low-power fields for the various MCF10A-derived cell lines are shown. Upper right is the cell size (area) comparison of HtrA1 transfection cell lines. Lower right is the histogram of the growth rate of the cell lines. Error bars represent the standard error of the mean. For cell size, MCF10A/siRNA1 and MCF10A/siRNA4 cell lines were significantly decreased cell area, while MCF10A/HtrA1 cell line was significantly increased that, compared to the control cell, MCF10A/HPVsh with t-test: ★★★, p-value $< 1 \times 10^{-8}$. For cell growth rate, at day4 and day6, the two HtrA1 knockdown cells, MCF10A/siRNA1 and MCF10A/siRNA4, grown significantly faster than the control cell, MCF10A/HPVsh with t-test: ★, p-value<0.01. Over-expression (~2X) of HtrA1 in the MCF10/HtrA1 cells had no effect on growth rate.
Table 5-2: Transcription Factors Inducing EMT

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<tr>
<th>Gene Symbol</th>
<th>ENM3</th>
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<th>ENM7</th>
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Table 5-2: Transcription Factors Inducing EMT (Continued)

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<th>ENM12</th>
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Table 5-2: Transcription Factors Inducing EMT (Continued)

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<th>Gene Symbol</th>
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The table above lists the transcription factors ENM3-ENM14 and their expression levels in EMT induction experiments. These factors are known to regulate EMT and are critical for the transition of cells from a mesenchymal to an epithelial phenotype.
A group of protein tyrosine phosphatases (PTP), receptor or non-receptor types, was also highlighted in the array data. One of this family proteins, PTP-Pez, has been reported to promote the EMT by inducing TGFβ signaling, which was associated with down-regulation of microRNA200 family members [118, 124] (Table 5-3).

The TGFβ signaling pathway can be a prominent driver of the EMT [123, 238]. The pleiotropic nature of TGFβ indicates that it has a complex role in tumor progression. On one side, TGFβ signaling pathways are often lost in hepatic, pancreatic, gastric and colorectal carcinomas [134]. On the other hand, in several cancer types high levels of TGFβ in patient serum is associated with poor prognosis [239-241]. In vitro, HtrA1 has been shown to bind to a broad range of TGFβ family proteins including Bmp4, Gdf5, TGFβs and activin in a GST-pulldown assay in mouse myoblast C2C12 cells [21]. However, we did not detect any consistent inverse correlation between HtrA1 levels and the expression of TGFβ1 in the microarray result (Table 5-4), and we did not find alterations in TGFβ1 from cell culture media in the various MCF10A cell lines. These data indicated that HtrA1 could be in a downstream position, or in a parallel pathway, for regulation of EMT.

Hypoxia is another event that can promote the EMT. Because of the uncontrolled cell proliferation, tumor cells actually suffer from hypoxia [154]. Under normoxic conditions, intracellular reactive oxygen species (ROS) are tightly controlled within narrow limits by redox regulatory mechanisms. Under hypoxic conditions, ROS can potentially activate HIF-1 through several signaling pathways, such as PI3K/AKT pathway, ERK/MEK pathway, and EGF/EGFR signaling [153]. The von Hippel-Lindau (VHL) is a tumor suppressor gene and its gene product pVHL is involved in HIF-1’s
regulation. It binds and degrades HIF α subunits in an oxygen-dependent manner [152]. The vascular epidermal growth factor (VEGF) is the major downstream effector of HIF-1 and it is the mediator of angiogenesis and vascular permeability [120].

The fibroblast growth factor (FGF) family genes include 22 members. They mediate cellular responses through binding and activation of the 4 different receptor tyrosine kinases (RTKs), FGFR1/2/3/4 [242]. FGFR2 gene is at human chromosome position 10q26, encoding FGFR2b and FGFR2c isoforms. FGFR2 plays oncogenic and anti-oncogenic roles in a context-dependent manner. Single nucleotide polymorphisms (SNPs) within intron2 of FGFR2 gene are associated with breast cancer through allelic up-regulation, while loss of FGFR2b signaling induces the EMT and dys-regulated ROS [243]. In addition, many AKT downstream substrates were also found altered in our result, such as GSK-3, MAP3K5, as well as members of the forkhead transcription factor family. Therefore, the entire angiogenesis and HIF pathway seem to be involved in the effects of HtrA1 down-regulation (Table 5-5). We further verified that EGFR and AKT were activated in HtrA1 knockdown cells by the phosphorylation-specific immunoblot analyses.
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<td><strong>Protein</strong></td>
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<td><strong>Protein</strong></td>
</tr>
<tr>
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<td>Serine/threonine protein kinase</td>
</tr>
<tr>
<td>ERK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>JNK</td>
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<tr>
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<td><strong>Protein</strong></td>
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<tr>
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<td>Hypoxia inducible factor 1-alpha</td>
</tr>
<tr>
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<td>CDK4</td>
<td>Cyclin-dependent kinase 4</td>
</tr>
<tr>
<td>MET</td>
<td>Met proto-oncogene, receptor tyrosine kinase</td>
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<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
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<td><strong>Protein</strong></td>
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<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<td>HIF-1alpha</td>
<td>Hypoxia inducible factor 1-alpha</td>
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<tr>
<td>NFκB</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
</tbody>
</table>
Recently, the EMT was related to be a trigger of cancer stem cell (CSC)-like phenotype [111]. CSC, which are believed to contribute to drug resistance, can self-renew and differentiate to generate the cellular heterogeneity of the original tumor [244]. For example, Al-Hajj et al. have isolated CD44+, CD24-/low breast cancer initiating cells with CSC-like properties [168]. Other commonly used markers include aldehyde dehydrogenase 1 (ALDH1), CD133, and ABCG2. ALDH1 has been identified as a specific marker for breast cancer-initiating cells [175]. Here, in the present study, we found in ~90% decrease of CD24 gene expression in HtrA1 knockdown cells compared with controls, coupled with an average 2 fold increase of CD44 expression (Table 5-6). We also detected a 1.2-3.6 fold expression changes in a number of ALDH1 subunits in the same HtrA1 knockdown cells (Table 5-6). Whether the CD24-/CD44+/ALDH1+ cells truly exist in HtrA1 knockdown MCF10A cells needs to be established, but our results provide a possible explanation for the increased drug-resistance related to decreased HtrA1 expression in breast cancer cells.

Before examination of the EMT phenotype in HtrA1 knockdown cells, we first confirmed the microarray transcript results for VIM and E-cad (CDH1) expression by QPCR analysis. Expressions of vimentin transcripts in HtrA1 knockdown cells (MCF10A/siRNA1 and MCF10A/siRNA4) were significantly increased 3 and 5, respectively, compared to levels in the irrelevant transfection control (MCF10A/HPVsh) cells (both p-value < 0.001). In contrast, overexpression of HtrA1 (MCF10A/HtrA1) induced a dramatic inhibition of vimentin expression (It was almost undetectable compared to the same control cell; p-value < 0.001) (Figure 5-2A).
In the opposite direction, expression of E-cad in HtrA1 knockdown cells, MCF10A/siRNA1 and MCF10A/siRNA4, was significantly decreased by 60% (p-value < 0.05) and 95% (p-value < 0.01), respectively, compared to that in the irrelevant control cell. HtrA1 overexpression resulted in elevated expression of CDH1 by > 5 compared to the same control cell (p-value < 0.05) (Figure 5-2B).

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**Figure 5-2: Confirmation of changes in gene expression of VIM and CDH1 by QPCR analysis.** Total RNAs were harvested from original MCF10A cell and MCF10A-derived cells, and analyses were performed 3 times. In HtrA1 knockdown cells, the mesenchymal marker gene, vimentin (VIM), significantly increased, while the epithelial marker gene, E-cadherin (CDH1), significantly down-regulated compared to the control cell, MCF10A/HPVsh. In the opposite direction, the HtrA1 overexpressing cell, MCF10A/HtrA1, showed dramatic increase of CDH1 and near loss of VIM expression, compared to the same control cell. Error bars represent the standard error of the mean. All p-values are a comparison to the irrelevant control.
C. Immunocytochemistry for EMT Markers

Next, we examined changes in expression of EMT-associated genes found in the microarray analysis using immunocytochemistry (ICC). We examined two markers of mesenchymal cells, VIM and N-Cad, and two markers of epithelial cells, CDH1 and KRT5/6/18 (KRT5/6 are myoepithelial proteins). We compared the staining results in two corresponding groups, VIM and KRT5/6/18, which are cytoskeleton components, and N-Cad and E-Cad which are adherin junction molecules (Figure 5-3).

Compared to control cells, HtrA1 knockdown cells showed dramatically increased VIM levels whereas HtrA1 overexpressing cells showed significantly decreased expression. The epithelial (and myoepithelial) cell biomarkers KRT5/6/18 showed large decreases in HtrA1 knockdown cells, while HtrA1 overexpressing cells showed slightly increased levels. In another group of comparison, N-Cad vs CDH1, we detected relatively weaker signals over all. This could be partially due to the parental MCF10A cell is a basal-like phenotype breast cell line, which typically have lower levels of epithelial biomarkers including CDH1. However, in the HtrA1 knockdown cells, MCF10A/siRNA1 and MCF10A/siRNA4, there was a notable increase in N-Cad signals, and overexpression of HtrA1 resulted in increased CDH1 signal, which was noted sporadically.
However, the expression levels of VIM, KRT5/6/18, N-cad, and CDH1 in those MCF10A-derived various cell lines are superior to compare quantitatively to confirm the ICC results, because based on the staining patterns, not all of the cells in one cell line express those marker genes and those genes expression levels in each cell were varied between cell lines. To this end, two experiments can be performed to quantify the EMT marker genes expression, immunoblotting analysis and fluorescence activated cell sorting (FACS). Whereas the immunoblotting analysis can show the overall expression level differences between cell lines, FACS has more advantages to demonstrate the differences among individual cells. By using the FACS, we expect to get the information of expression changes of certain EMT marker gene between cell lines, how many cells in one cell line express that gene, and what is the expression level of individual cells.
Figure 5-3: Cell Staining for EMT Markers. Representative micrographs from vimentin, KRT5/6/18, N-cad, and E-cad immunofluorescence stains are shown. Two HtrA1 knockdown cells, MCF10A/siRNA1 and MCF10A/siRNA4, HtrA1
D. Migration and Invasion Test

Since increased cell motility is one of the characteristics of the EMT, we examined the migration and invasion ability of the MCF10A-derived cell lines in a transwell assay. Uncoated transwell inserts were used to test cells migration ability, whereas inserts coated with 1x Basement Membrane Extract (BME) were used to determine the cells invasion capability. Results indicated that, compared to MCF10A/HPVsh irrelevant control cell line, one of the HtrA1 knockdown cell 10A/siRNA4 significantly increased its migration ability (p-value<0.01), while HtrA1 overexpressing cell line significantly decreased it (p-value<0.01). Both of the two HtrA1 knockdown cells tested, MCF10A/siRNA1 and MCF10A/siRNA4, significantly increased cell invasion ability compared to the irrelevant control cell (p-value<0.01) (Figure 5-4).
E. MicroRNA Array Analysis

There are several approaches for studying genome-wide microRNA (miR) profiles. One of the most commonly used is miR oligo array [245]. The experimental design of these miR arrays is essentially similar to gene expression arrays.

The role of miRs in the EMT is an area of active investigation. To examine the effects of altered HtrA1 expression on miR profile, we used the various MCF10A-deried cell lines and the Illumina v2 MicroRNA Assay Beadchip platform. 1146 genes are

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**Figure 5-4: Motility changes for MCF10A-derived cell lines.** The motility of HtrA1 overexpressing and knockdown cells was tested with migration and invasion assay. Cells were plated in the insert of the transwell: passing through the permeable supports either with (invasion, the red bar) or without (migration, the blue bar) a layer of coated Basement Membrane Extract (BME) were determined. Error bars represent the standard error of the mean. All p-values are in comparison to the irrelevant control cell MCF10A/HPVsh by t-test: ★, p-value<0.01.
represented on the chip. For the comparison, we used a 1.4x cut-off, with p-values less than 0.05. Compared to MCF10A/vector control cell line, the MCF10A/siRNA1, siRNA2, siRNA3, and siRNA4 cell lines showed 53, 16, 32, and 39 significantly changed miRs, respectively. Several identified miRs showing significant changes in HtrA1 knockdown cells have been impacted in breast cancer biogenesis (Table 5-7). MiR-200 family members (has-miR-429, has-miR-200a (a*), has-miR-200b (b*), and has-miR-141, highlighted as blue background in the table) were the most significantly decreased group of miRs in HtrA1 knockdown cells, showing decreases ranging from 20% to more than 60%. This group of miRs may directly regulate the EMT transcription factors, such as ZEB1, ZEB2, or through their target genes. For example, PTPN12 is one of their known targets. In the reverse direction, has-miR-328 and has-miR-10a were increased to more than 2-fold in the MCF10A/siRNA cell lines. Their inhibition targets include HOXB8, HOXA1, Sox-21, PLXDC2 which all have known tumor suppressor functions.

<table>
<thead>
<tr>
<th>miRNA</th>
<th>siRNA1</th>
<th>siRNA2</th>
<th>siRNA3</th>
<th>siRNA4</th>
<th>Average</th>
</tr>
</thead>
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<tr>
<td>hsa-miR-429</td>
<td>0.058</td>
<td>0.618</td>
<td>0.695</td>
<td>0.107</td>
<td>0.369</td>
</tr>
<tr>
<td>hsa-miR-200a</td>
<td>0.169</td>
<td>0.657</td>
<td>0.766</td>
<td>0.201</td>
<td>0.448</td>
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<td>hsa-miR-200a*</td>
<td>0.397</td>
<td>0.806</td>
<td>0.936</td>
<td>0.390</td>
<td>0.632</td>
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<tr>
<td>hsa-miR-200b</td>
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<td>0.631</td>
<td>0.629</td>
<td>0.163</td>
<td>0.440</td>
</tr>
<tr>
<td>hsa-miR-200b*</td>
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<td>0.538</td>
<td>0.664</td>
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<td>0.800</td>
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<td>hsa-miR-200c*</td>
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<td>0.737</td>
<td>0.923</td>
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<td>0.520</td>
<td>0.634</td>
<td>0.530</td>
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</tr>
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</table>
F. Activation of EGFR/AKT Pathway

As one of the known inducers of the EMT, the EGFR/AKT signaling pathway was identified as significantly changed in the EMT changes induced by HtrA1 down-regulation. By immunoblot analysis for phosphorylated EGFR and AKT, we found that both of them were activated in HtrA1 knockdown cells. Newly formed phosphorylated EGFR and increased level of phosphorylated AKT were found in the cytoplasmic fractions of MCF10A/siRNA4 and MCF10A/siRNA1 cells (Figure 5-5). Moreover, in the nuclear fractions of these two HtrA1 knockdown cell lines (particularly MCF10A/siRNA4), phosphorylated AKT was also observed.

These results confirm a link between EGFR/AKT and HtrA1 expression: Down-regulation of HtrA1 activated the EGFR in the MCF10A-derived cell lines, and enhanced the activation of its downstream effector, AKT.

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<tr>
<th>hsa-miR-328</th>
<th>2.637</th>
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<th>2.060</th>
<th>3.373</th>
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<td>2.079</td>
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<td>hsa-miR-34c-3p</td>
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<td>0.552</td>
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<td>hsa-miR-627</td>
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<td>0.473</td>
<td>0.485</td>
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<td>hsa-miR-188-5p</td>
<td>0.461</td>
<td>0.452</td>
<td>0.540</td>
<td>0.521</td>
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HtrA1 has been shown to inhibit signaling mediated by TGFβ family proteins in mouse myoblast C2C12 cells [21] and TGFβ signaling is believed to be a prominent
driver for the EMT in a number of settings [123, 238]. Therefore, we compared the secretion levels of TGFβ1 by the MCF10A-derived transfected cell lines in medium from cell cultures. Samples included the parental MCF10A cell, the Flp-In transfection host cell MCF10A/Flp, a plasmid vector control cell MCF10A/Vector, a cell line expressing an irrelevant siRNA (MCF10A/HPVsh), the HtrA1 overexpressing cell line MCF10A/HtrA1, all four HtrA1 knockdown cell lines (MCF10A/siRNAs), and a cell culture medium blank. We found no consistent differences in the levels of secreted TGFβ1 in HtrA1 knockdown cells. Control cell lines averaged 728.372pg/ml, whereas the four HtrA1 knockdown cell lines averaged 731.307pg/ml.

**H. Doxorubicin-induced Cytotoxicity**

HtrA1 could potentially play a pivotal role in regulating Doxorubicin (Dox) sensitivity in human breast tumors [10]. Hence, we examined two groups of breast epithelial cells for response to Dox. First, we compared the cell survival rates between breast cancer cell lines and two non-tumorigenic breast epithelial cell lines (Figure 5-6A). Four cancer cell lines, MCF7, M4A4, MDA-MB-231, and MDA-MB-468, demonstrated much higher survival rates compared to two non-tumorigenic breast epithelial cell lines, MCF12A and MCF10A, at each concentration of Dox. We have shown that HtrA1 was highly expressed in these two non-tumorigenic cell lines and was dramatically decreased or almost lost in these four cancer cell lines at both the mRNA and protein levels (Figure 3-1, 6). Therefore, these results support clinical data showing that higher expression
levels of HtrA1 corresponded to a higher response rate to Dox in human breast tumors [10].

To further investigate the potential role of HtrA1 in Dox-sensitivity, we also used the various MCF10A-derived cell lines (overexpression and knockdowns), for sensitivity to Dox (but at lower concentrations of Dox; see Figure 5-6B). We found that compared with the parental MCF10A cells and the various control cell lines, HtrA1 knockdown cells were slightly more sensitive to Dox, whereas HtrA1 overexpression cell were slightly less sensitive, at doses below 0.05 µg/ml. The differential sensitivity to Dox at higher concentration (0.25 µg/ml) was low between HtrA1 knockdown cells and HtrA1 overexpression cells. These data thus seem contradictory to the previous results in Figure 5-6a. However, this group of cells was derived from a non-tumorigenic breast cell line MCF10A, and additional results (see Chapter 6) also indicate that overexpression of HtrA1 could protect MCF10A-derived cell lines from ROS-induced DNA damage, whereas HtrA1 knockdown activated the ATM-initiated DNA damage response pathway. This result might partially explain these results, since Dox is a DNA damaging drug [246].
Figure 5-6: Doxorubicin cytotoxicity on human breast cells and HtrA1 transfected MCF10A cells. Cell survival was measured by Dojindo cell counting kit-8 (CCK-8) after 48hr of drug treatment. A serial of concentrations of doxorubicin (dox) were treated to individual cell line. Sample size is seven for each concentration. Results are expressed as means ± SE. The y-axis represents portion of surviving cells. A. Comparison between human breast cancer cell lines, MCF7, M4A4, MDA-MB-231, MDA-MB-468 and two normal cells, MCF12A and MCF10A. B. Comparison between MCF10A-derived various transfected cell lines. Experiment was repeated once to confirm the tendency.
4. Discussion

Growth of a primary breast tumor itself is rarely fatal, but the cell growth at distant sites after metastasis results in mortality. The formation of the metastasis involves multiple steps including migration out of the primary tumor, invasion into the vascular (or lymphatic) system, survival in the blood stream, exit out of the blood stream and survival and proliferation at a secondary site [157]. Several studies using in vivo mouse models have observed that the EMT is involved in breast cancer metastasis [247, 248].

Studies in this chapter showed that down-regulation of HtrA1 in a series of cell lines derived from the MCF10A cell line decreased the expression of epithelial cell markers such as CDH1 and KRTs, but increased the expression of mesenchymal genes such as vimentin and N-cad. Decreased expression of HtrA1 also stimulated cell growth, migration and invasion capabilities. All these phenotypes suggest that decreased expression of HtrA1 triggers the EMT. We further investigated several mechanisms of EMT induction in this model. In HtrA1 knockdown cells, microRNA 200 family members were dramatically down-regulated; the EGFR/AKT signaling pathway was activated; and no changes of TGFβ1 were observed.

Cancer therapy is often associated with two forms of drug resistance, de novo or acquired. EMT and EMT-induced CSCs are suggested as one of the causative mechanisms of acquired drug resistance based on the environment [171]. For example, higher levels of E-cadherin are related to sensitivity to EGFR kinase inhibitors, while
mesenchymal cells are generally more drug resistant [234]. Cancer cells with stem cell features, CD44\textsuperscript{high}/CD24\textsuperscript{low}, are resistant to neoadjuvant chemotherapy and HER2 pharmacological inhibition [180]. Moreover, basal subtype mesenchymal-like breast cancers show higher mortality rates. However, several studies also suggest that in breast cancers, basal (mesenchymal-like) tumors are initially more sensitive to neoadjuvant chemotherapy than luminal (epithelial) tumors [249-251]. Therefore, it is possible that epithelial-like cancers are initially more sensitive to targeted agents, such as EGFR and HER2 antagonists, while mesenchymal cancers are more sensitive to DNA damaging agents such as doxorubicin and the mesenchymal-like cancers are more prone to acquire drug resistance [171].

We summarized the commonly used breast cancer chemotherapeutic reagents and their working mechanisms in the following table (Table 5-8).

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Mechanisms</th>
<th>References</th>
</tr>
</thead>
<tbody>
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<td>Doxorubicin</td>
<td>DNA damaging reagent, inhibition of both topoisomerase enzyme and DNA synthesis</td>
<td>Kalyanaraman \textit{et al}, 2002</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>DNA and protein cross-linker</td>
<td>Chien \textit{J et al}, 2006</td>
</tr>
<tr>
<td>Trastuzumab</td>
<td>HER2-targeted monoclonal antibody</td>
<td>Germano \textit{et al}, 2009</td>
</tr>
<tr>
<td>Lapatinab</td>
<td>Dual HER2 and EGFR tyrosine kinase inhibitor</td>
<td>Konecny \textit{et al}, 2008</td>
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</table>
Besides the EMT, many other factors could contribute to drug resistance in cancer cells, including changes in the extracellular microenvironment, changes in the drug target, increased repair of DNA damage or increased tolerance of DNA damage, disruptions in apoptotic signaling pathways, and expression of proteins and enzymes associated with drug resistance [252, 253]. To date, the most well studied mechanisms of chemoresistance are related to ATP-binding cassette membrane transporter family members, such as P-glycoprotein (P-gp), multidrug-resistant protein 1 (MRP1), and breast cancer resistance protein (BCRP). These transporters are found in normal mammalian cells, where they serve as protectors from excessive extracellular and intracellular concentrations of xenobiotics and toxins [252]. P-gp is encoded by the MDR1 gene, and its expression level has a strong correlation with the extent of drug resistance to either doxorubicin or paclitaxel in an assay of 359 freshly resected specimens of breast carcinoma [254]. In addition to P-gp, many other transporter proteins in the multidrug resistance protein (MRP) family are associated with chemoresistance. For instance, MRP1 confers resistance to anthracyclines, antifolates, and vinca alkaloids

<table>
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<tr>
<th>Compound</th>
<th>Description</th>
<th>Reference</th>
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<tr>
<td>Salinomycin</td>
<td>Antibiotic, preferentially kill breast cancer stem cells</td>
<td>Gupta et al, 2009</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>Folic acid antagonist</td>
<td>Zhanglin Ni et al, 2010</td>
</tr>
<tr>
<td>Mitoxantrone</td>
<td>Antineoplastic, immunomodulatory agent</td>
<td>Scott et al, 2004</td>
</tr>
<tr>
<td>Etoposide</td>
<td>Topoisomerase II inhibitor, DNA damaging reagent</td>
<td>Teixeira AC et al, 2009</td>
</tr>
<tr>
<td>Vincristine</td>
<td>Microtubule-targeting agent</td>
<td>Risinger et al, 2010</td>
</tr>
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</table>
MRP2 is associated with resistance to methotrexate, etoposide, cisplatin, and doxorubicin in human cancer cell lines [257, 258]. BRCP, also known as mitoxantrone resistance gene, has been shown expressed in a range of cultured breast cancer cell lines such as MCF7 [259]. This protein may confer resistance to mitoxantrone, anthracyclines, methotrexate, and topoisomerase I inhibitors [260, 261].

In our microarray analysis results, we detected an increase of CD44 and ALDH1 gene expression and a decrease of CD24 gene expression in HtrA1 knockdown MCF10A cells. The CD44\textsuperscript{high}/CD24\textsuperscript{low}/ALDH1\textsuperscript{+} corresponds to the breast cancer stem cell marker phenotype. Therefore, we might also explore the potential contribution of CSCs involved in induction of the EMT phenotype, which occurs with HtrA1 down-regulation. First, the subpopulation of cells carrying CD44\textsuperscript{high}/CD24\textsuperscript{low} marker genes, which might constitute a very low percentage of the whole population, need to be identified, which could be performed via flow cytometry. Sorted cells then need to be characterized by QPCR analysis for the genotype of CD44\textsuperscript{high}/CD24\textsuperscript{low} and probably several other stem cell marker genes, such as ALDH1, CD13, EpCAM, compared to the rest of the cells in the population. If identified, CD44\textsuperscript{high}/CD24\textsuperscript{low} cells could be tested for mammosphere-forming ability, chemosensitivity, and even tumorigenic potential SCID mice.

Better understanding of the EMT process may in time contribute to the development of new therapeutic strategies, which are based on inhibition of the expression or function of EMT-inducing factors in malignant carcinomas. In vitro models for studying EMT have limitations, though. For example, it is common to have incomplete EMT phenotypes since the EMT is very sensitive to culture conditions, including substrate, the presence of serum, and even cell density [105]. In the future, the
development of appropriate in vivo models as well as extensive studies with human tumors can help to clarify the involvement of HtrA1 down-regulation induced EMT in tumorigenesis and tumor progression.
Chapter 6

Evaluation for the Role of HtrA1 in DNA Damage Response

1. Introduction

Potential HtrA1 function that was indicated in our gene array analysis (chapter 4) was the involvement in the DNA damage response (DDR) network.

The nature of genotoxic stress is a challenge for cell survival. In order to ameliorate the damage to DNA, intact DDR pathways are critical for arresting cells at various checkpoints to provide time for repair, and/or to decide to whether to initiate apoptosis if the damage is overwhelming. Responses involved in this network may include transcriptional activation, cell cycle arrest, DNA repair, apoptosis, and cellular senescence [262].

The ataxia telangiectasia-mutated (ATM) gene/protein plays a central role in the DDR network. ATM is a nuclear protein kinase. ATM exists in the nucleus as dimers associated with protein phosphatase 2A (PP2A) under normal physiological conditions [263]. However, when a DNA double-strand break (DSB) occurs, ATM will dissociate from PP2A and become activated through autophosphorylation at serine-1981 [264]. Activated ATM then initiates a series of downstream phosphorylation events.

Dynamically, the MRE11-NBS1-RAD50 (MRN) complex first binds to the damaged DNA site, which then recruit ATM. Recruitment of MDC1 follows, and its binding to chromatin initiates phosphorylation of histone H2AX, and then recruitment of 53BP1
occurs. Following the generation of a DSB, ATM is activated and phosphorylates Ser-139 of H2AX (designated as γH2AX) in a megabase chromatin domain flanking the lesion [265]. The phosphorylation status of H2AX is one of the most important DDR early events, which can amplify the damage signal and help recruit other response molecules to the site, thus transducing the signal to downstream signaling pathways [266]. γH2AX reaches a plateau within 30 min of insult and it can be visualized as foci using γH2AX antibody, which is used as a universal biomarker of DNA double-strand break [265]. γH2AX holds the broken DNA ends in close proximity and recruits other DNA repair molecules to the damage site [267]. Radiation and chemotherapeutic agents, e.g. X-ray and TOPO II poisons, are known to cause DSBs and they induce γH2AX foci in various cell types [268]. This is a cyclic process that amplifies the damage signal by repeated protein-protein interactions and recruitment of additional molecules [194].

The crucial step for understanding the entire DDR network is to understand the early sequential events [269] (Figure 6-1). The generally agreed hierarchy model of this process includes the first level sensors and the second level transducers. The nuclear protein kinases, ATM and ATR (ATM and Rad3-related) are major sensors of DNA damage. While ATR and ATRIP (ATR-interacting protein) response to single-strand DNA (ssDNA), ATM and its regulator MRN (Mre11-NBS1-Rad50) complex sense double-strand breaks (DSBs) [270]. CHK1 and CHK2 are the two key mediators at this level. They could direct different downstream effects through different third level effectors. For example, whereas CHK2 is primarily involved in DDR-induced apoptosis, CHK1 is more related to the cell cycle checkpoint. Specifically, CHK1 phosphorylates the phosphatase CDC25A to target it for ubiquitin-dependent degradation. Thus CDC25A
can no longer activate CDK1 and CDK2. Cells will be arrested in late G1, S, and G2 phases [271]. Besides CHK1 and CHK2, p53 is another pivotal transmitter in the response to DNA damage. Depending on the cellular context, activated p53 could act as a transcriptional activator and/or suppressor to dictate either cell growth arrest and repair, or apoptosis if the damage is overwhelming.

Figure 6-1: ATM/ATR-dependent DDR pathway. Responding to the DNA damage, two core kinases ATM and ATR are activated and recruit the sensors, such as the MRN complex, 53BP1, MDC1 in the ATM pathway and RPA in the ATR pathway; transducers, such as CHK1, CHK2, p53, and BRCA1; and effector proteins, and finally regulate the cell cycle checkpoint, DNA repair, and apoptosis. Figure was slightly modified from [269].
Double-strand breaks (DSBs) are highly deleterious lesions in genomic DNA. They can be generated by ionizing irradiation and a variety of chemical agents, such as topoisomerase II (TOPO II) poisons, heavy metal ions and reactive oxygen species (ROS) [268].

In order to test the involvement of HtrA1 in DDR, we used acute oxidative stress induced by hydrogen peroxide. The “stressed” status of cells was measured by dichlorofluorescein (DCF) assay. The corresponding HtrA1 mRNA and protein levels in the various MCF10A-derived cell lines were examined by QPCR and immunoblot analyses. As the important early events of ATM-initiated DDR, phosphorylations of ATM, CHK1, and CHK2, were characterized by Western analyses before and after the H$_2$O$_2$ treatments. H2AX is another early substrate that becomes phosphorylated by ATM following formation of DSBs. We looked at γH2AX foci in all the HtrA1 transfected cells with and without H$_2$O$_2$ treatment.

Overall, data in this chapter clearly suggests a role of HtrA1 in control of the DDR.

2. Materials and Methods

A. Quantitation of Cellular Oxidative Stress by DCF Assay and Kill Curve

Cells were washed with PBS and then incubated with 100µM DCFH_DA in medium in 5% CO$_2$ at 37C for 30 min. After DCFH-DA was removed, cells were washed and treated with a series of concentrations of H$_2$O$_2$ in medium. The fluorescence of cells
was immediately measured using a plate reader (485nm for excitation, and 530nm for emission). Data points were taken every 30 min for 2 hr.

When applied to intact cells, the nonionic, nonpolar DCFH-DA (2',7'-dichlorofluorescin diacetate) crosses cell membranes and is hydrolyzed enzymatically by intracellular esterases to nonfluorescent DCFH. In the presence of reactive oxygen species (ROS), DCFH is oxidized to highly fluorescent dichlorofluorescein (DCF). Therefore, the intracellular DCF fluorescence can be used as an index to quantify the overall OxS in cells. After withdrawing treatment, cells were exposed to normal culture medium for another 22hr. Cytotoxicity curves were measured with the cell counting kit-8 (CCK-8) from Dojindo Molecular Technologies, INC.

Experiments were performed at least three independent times.

**B. QPCR and Western Blotting for Detection of HtrA1 Response to Acute ROS**

The same amount of cells for each cell line was plated (2x10^4 cells/cm^2) the day before each experiment. The next day, cells were treated with a series of concentrations of H_2O_2 in medium (0, 100, or 200 µM) for 2 hours, and then switched back to normal medium for another 22 hours. On the following day, total RNA was extracted with Qiagen RNeasy mini kit (cat. #74904). Quantitative real-time PCR was performed with the QuantiTect Probe RT-PCR kit (Qiagen, Cat. # 204443) on Stratagene Mx4000 QPCR systems (Agilent Technologies). Each cell line was treated in triplicate for each concentration of H_2O_2 and each RNA sample was analyzed in triplicate for QPCR. 200 ng of total RNA for each sample was used in each reaction. All quantification data were normalized to TBP, which acts as an internal control. The following primers and probe
were used for human HtrA1, 5’-TTGTTTCGCAAGCTCAGTT-3’ (forward), 5’-ACGTGGGCATTTCGACCA T-3’ (reverse), 5’-FAM-TCTAAACGAGAGGTGCTGGCTAGT-BHQ-3’ (probe); for human TBP, 5’-CACGGCACTGATTTTCAGTTCT-3’ (forward), 5’-TTCTTGCTGCCAGTCTGGACT-3’ (reverse), 5’-HEX-TGTGCACAGGAAGCTGGAAGA-BHQ-3’ (probe).

Results were expressed as Mean ± SE.

In parallel, whole cell lysates were harvested for immunoblot analysis. Samples were separated by electrophoresis using 4-12% SDS-PAGE. After transferred at 40V for 2 hr, membranes were blocked with 6% milk in TBST for 1.5hr at RT. Primary antibody, mouse monoclonal anti-human HtrA1/Prss11 (R&D Systems, Cat. No. MAB2916), 1:500 dilution, was incubated overnight at 4C. Secondary antibody was anti-mouse IgG, HRP-linked (Cell Signaling Technology, #7076). As loading control, β-actin mouse monoclonal antibody from Santa Cruz (#SC-47778) was applied and for nuclear fraction, we used rabbit polyclonal anti-DEK antibody (Aviva Systems Biology, #P100637).

C. Immunoblotting for Detection of Early Events in DNA Damage Response.

The same amount of cells for each cell line was plated (2x10^4 cells/cm^2) the day before experiment. The next day, cells were treated with 0 or 100 µM of H_2O_2 in medium for 2 hours. Then whole cell lysates were harvested and analyzed with the following antibodies: mouse monoclonal anti-Chk1 (Cell Signaling Technology, #2360), rabbit monoclonal anti-phospho-Chk1 (Tyr345) (Cell Signaling Technology, #2348), mouse monoclonal anti-Chk2 (Cell Signaling Technology, #3440), rabbit monoclonal anti-phospho-Chk2 (Thr68) (Cell Signaling Technology, #2197) and mouse monoclonal anti-
phospho-ATM (ser1981) (Upstate, #05-740). As loading control, β-actin mouse monoclonal antibody from Santa Cruz (#SC-47778) was applied. Secondary antibodies were from Cell Signaling Technology, anti-mouse IgG, HRP-linked, #7076; anti-rabbit IgG, HRP-linked, #7074.

D. Phosphorylated-γH2AX Staining Protocol

Cells were plating at 4x10^4 cells/cm² on the chamber slides the day before staining. 24 hours later, cells were treated with H₂O₂ at 200 µM for 1 hr. Meanwhile, etoposide at 25 µg/ml was used as a positive control for forming DNA DSBs. After treatment, cells were fixed with 10% neutral buffered formalin for 20 min at RT. After rinsing with PBS, cells were permeablized in PBS/0.2% Triton-X for 10 min at RT, followed by blocking with 2.5% goat serum in PBS/0.1% trinton-X for 1 hr at RT. Biotin-conjugated anti-phospho-Histone H2AX (ser139) (Millipore #16-193) antibody was diluted in the same blocking buffer and incubated with samples at 4°C overnight. On the following day, cells were washed with PBS/0.2% Triton-X 3 times for 3 minutes and incubated with tetramethylrhodamine-conjugated streptavidin antibody (Molecular Probes #S870) for 30 min at RT, and finally washed with PBS/0.2% Triton-X 3 times for 3 minutes, and then examined microscopically. Experiments were performed at least three independent times.

E. Flow Cytometry for Apoptosis

Three plates (1x10^4 cells/cm²) of cells for each cell line were incubated with 50 µM of H₂O₂ in medium for 24 hours before collecting and follow the ApoAlert Annexin
V kit manufacture’s instructions (Clontech, #630109). Briefly, cells were labeled with Annexin V-FITC (20 µg/ml) and Propidium Iodide (50 µg/ml) for 15 min at RT in the dark. Then apoptotic cells were quantified by FACSscan flow cytometry (BD Biosciences). As a positive control for apoptosis, cells were treated with 50 nM staurosporine (Sigma, #S6942) for 24 hours. Results are expressed as Mean ± STDEV.

F. NFκB and 4HNE ICC After H₂O₂ Treatment

2 x 10⁴ cells/well for each sample, multiple wells for each MCF10A-derived transfection cell line, were plated on chamber slides the day before experiment. On the experiment day, for each cell line, cells were treated with either N/A (as controls) or 100 µM H₂O₂ for 2 hr before fixed. After permeablized and blocked, cells were incubated with primary antibody at 4°C overnight. On the following day, cells were washed and incubated with secondary antibody for 1 hr at RT. After the final wash, DAPI was used to counterstain cells for 5 min, and slides were mounted and examined.

The primary mouse anti-NFκB p65 antibody was from BD Transduction Laboratories (cat# 610869), and was tested with 1:50 and 1:100 dilutions. The primary mouse monoclonal antibody for 4HNE was kindly provided by Dr. Weisz’s lab, and was tested with 1:500 and 1:1000 dilutions. The fluorescent secondary antibody was Cy3-Donkey anti-mouse, and used as 1:1000 dilution.
3. Results

A. Gene Array Results for ATM Network

Table 6-1 is a summary of ATM-network genes showing changes in our array. They have varied fold(s) changes in transcript levels. However, to verify the activation of the DDR pathway requires further testing of protein phosphorylation states.
B. Cellular Response and HtrA1 Response to Acute Oxidative Stress

To investigate the potential role of HtrA1 in DDR, acute OxS treatment, induced by hydrogen peroxide, was applied to all the various MCF10A-derived cell lines. First, the overall ROS in cells within the 2 hr H$_2$O$_2$ treatment were measured using the DCFH as described. H$_2$O$_2$ cytotoxicity curves were also collected (Figure 6-2 is an example result from the MCF10A cell line). Treatment with 100 µM-200 µM H$_2$O$_2$ for 1-2 hr appeared to be the optimum range for inducing OxS in the cell lines. The series of HtrA1 transfected cell lines (MCF10A/HPVsh, MCF10A/HtrA1, MCF10A/siRNA1-4) showed similar levels of ROS and cell survival rates (data not shown).

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**Figure 6-2: Quantitation of cellular oxidative stress in H$_2$O$_2$-treated cells.** Results of MCF10A cell are shown as examples for all the MCF10A-derived various cell lines. On the left, 2hr quantitation of cellular ROS was measured by dichlorofluorescein assay. Different concentrations of H$_2$O$_2$ were monitored every 30min in the first 2hr. On the right, different concentrations of H$_2$O$_2$ kill curve was determined after changing back to normal culture medium for another 22hr. Similar results of both assay were obtained from all other MCF10-derived cell lines.
In parallel, HtrA1 expression levels in response to the acute H$_2$O$_2$ treatment were also examined in all cell lines. At the mRNA level, QPCR results showed significantly elevated HtrA1 expression level in MCF10A/HtrA1 overexpressing cell line, whereas the other cell lines did not show any obvious induction (Figure 6-3a). HtrA1 protein levels seem relatively constant in all the various cell lines in immunoblot analysis (Figure 6-3b).

**Figure 6-3: HtrA1 expression levels in response to H$_2$O$_2$ treatment.** To test the HtrA1 response to the acute oxidative stress treatment in HtrA1 transfected cell lines, QPCR and Western blot analyses were performed. In panel A, QPCR result is
showing the HtrA1 mRNA levels in cells after the 2 hr treatments with various concentrations of H$_2$O$_2$ followed by 22 hr recovery. The MCF10A/HtrA1 cell line showed a major increase in HtrA1 expression after treatment, although the induction may come from the transfected construct, which driven by human CMV promoter. Error bars represent the standard error of the mean. In panel B, the corresponding protein levels of HtrA1 in various cells were detected using the HtrA1 monoclonal antibody (R&D Systems). Whole cell lysates were prepared 24hr after the treatment. HtrA1 protein levels with or without the treatment in individual cell line did not show any significant difference.

**C. Phosphorylation of Proteins in DDR Early Detection**

Immunoblotting results showed that ATM was activated (phosphorylated) in HtrA1 knockdown cells even without acute H$_2$O$_2$ treatment. Phosphorylated ATM bands were observed in MCF10A/siRNA1 and MCF10A/siRNA4 cells but not in MCF0A/HPVsh irrelevant control cell line or the MCF10A/HtrA1 overexpressing cell line (Figure 6-4). This indicated that HtrA1 might play a role in maintaining DNA integrity and stability.

When treating the same group of cells with acute H$_2$O$_2$ (100 µM, 2 hr), ATM, CHK1 and CHK2 in all cells were phosphorylated, with the exception of CHK1 in the MCF10A/HtrA1 cell line. This further indicated that HtrA1 might be associated with cell cycle checkpoints via interaction with CHK1.
Figure 6-4: Immunoblot analysis for phosphorylation of components important in early DNA damage response pathway. Activations of ATM, CHK1, and CHK2 were detected with phosphorylation-specific antibodies. Cell lines are as indicated on the right, and (-) and (+) indicate acute treatment with H$_2$O$_2$. Phosphorylated ATM was observed in the absence of any treatment in both MCF10A/siRNA cell lines examined. Following H$_2$O$_2$ treatment, ATM, CHK1, and CHK2 all showed activation in MCF10-derived cell lines, except the overexpressing MCF10A/HtrA1 cell line.
D. Phosphorylation of DNA Double-Strand Break Marker γH2AX

From the microarray results, ATM transcripts levels were increased ~ 2X in the HtrA1/siRNA cell lines. To further test whether ATM-initiated DDR is functional activated in the HtrA1 knockdown cells, we treated the various MCF10A-derived cell lines with acute H₂O₂, which induces DNA damage including DSBs. As a positive control, the MCF10A cell line was treated with etoposide. Etoposide is the topoimerase II inhibitor and is an inducer of DSB throughout the cell cycle [272]. Nuclear γH2AX foci were readily detected in the MCF10A/siRNA4 cell line (Figure 6-5A). Staining results also showed that the HtrA1 overexpressing cell, MCF10A/HtrA1, significantly decreased γH2AX foci compared to controls. The untreated parental MCF10A cell line did not show any γH2AX signal. These results indicate that HtrA1 appear to protect cells from oxidative stress-induced DNA damage, whereas decreased levels of HtrA1 expressions predispose cells to more severe damage.

We also checked the DSB foci in all the HtrA1 transfected cells without any H₂O₂ treatment (Figure 6-5B). We detected a slight increase of γH2AX foci in HtrA1 knockdown cells compared with parental MCF10A cell and the irrelevant control cell, MCF10A/HPVsh, while HtrA1 overexpressing MCF10A/HtrA1 cell line was completely negative for the γH2AX foci.
Figure 6-5A: Immunofluorescence staining for phosphorylated H2AX foci (γH2AX) (with 200µM, 1hr H2O2 treatment). Representative micrographs show staining for cell lines as indicated with detection of γH2AX using an antibody specific for phosphorylated serine139 on the left side (red). Over-expression of HtrA1 blocks formation of γH2AX foci following acute treatment with H2O2, whereas foci formation is substantively increased in MCF10A/siRNA4 cells. The right field shows staining with Dapi (blue). Experiment was performed at least three independent times.
Figure 6-5B: Immunofluorescence staining for phosphorylated H2AX foci (γH2AX) (without H₂O₂ treatment). Representative micrographs show staining for cell lines as indicated with detection of γH2AX using an antibody specific for phosphorylated serine139 on the left side (red) without acute H₂O₂ treatment. Slightly increased γH2AX foci were tested in two HtrA1 knockdown cells, MCF10A/siRNA1 and 4 compared with original MCF10A and the irrelevant control MCF10A/HPVsh cells, while HtrA1 over-expression MCF10A/HtrA1 cell was completely negative for the antibody. MCF10A cell treated with H₂O₂ (200µM, 1hr) is the positive control for the experiment. The right field shows staining with Dapi (blue). Experiment was performed at least three independent times.
E. Flow Cytometry for Apoptosis

To test the possible role of HtrA1 in DDR connected to cell apoptosis, flow cytometry analysis was performed to the various MCF10A-derived cell lines. By averaging the percentages of apoptotic events in MCF10A and MCF10A/HPVsh cell lines as the control, MCF10A/HtrA1 cell has similar apoptotic rates for both early and late apoptosis, while the two knockdown cell lines, MCF10A/siRNA1 and MCF10A/siRNA4, has higher apoptotic rates for both events. However, the large standard deviations of the MCF10A/siRNA4 cell for both events made the results insignificant for this cell line.

| Table 6-2 Apoptotic Rates After H\textsubscript{2}O\textsubscript{2} Treatment |
|-----------------------------|---------------------------|---------------------------|
| Cell Lines                  | Early Apoptosis (\% events ± STDEV) | Late Apoptosis (\% events ± STDEV) |
| control                     | 2.572 ± 1.255             | 3.817 ± 1.051             |
| MCF10A/HtrA1                | 2.197 ± 1.210             | 4.187 ± 0.206             |
| MCF10A/siRNA1               | 3.420 ± 0.602             | 5.467 ± 0.620             |

F. NF\textsubscript{κ}B and 4HNE ICC After H\textsubscript{2}O\textsubscript{2} Treatment

To investigate the role of HtrA1 in protection from ROS-induced damages, NFκB and 4-hydroxynonenal (4HNE) were tested as protein and lipid damage markers in cells, respectively, following acute oxidative stress treatment.
NFκB is an inducible and ubiquitously expressed transcription factor. It is involved in regulating genes related to cell differentiation, survival, inflammation, and growth. NFκB is in the configuration of a dimer of two members of the NFκB/Rel/Dorsal (NRD) family of proteins, and the classical dimer is composed of p50 (also called NFκB1) and p65 (also called RelA) with only p65 having a transactivation domain [273]. In un-stimulated cells, NFκB is sequestered in the cytoplasm by interacting with a member of the inhibitory (IκB) family. After stimulation, IκB is dissociated and NFκB will translocate into nucleus and bind to κB regulatory elements [181].

We did not detect the nuclear translocation of NFκB in any of the various MCF10A-derived cell lines after H₂O₂ treatment (100 µM, 2 hr) using immunofluorescence cell staining with the NFκB p65 antibody. These preliminary results suggest that the H₂O₂ treatment in this experiment did not activate NFκB, although transient activation could have been missed.

4HNE is a lipid peroxidation product, which is widely used as the marker of chronic OxS. In an attempt to examine the role of HtrA1 in lipid oxidation, we incubated the MCF10A-derived various cell lines with 4HNE antibody after acute H₂O₂ treatment. However, we did not observe any 4HNE signal via ICC. The negative results could be due to the un-mature technique, or the inappropriate biomarker for acute oxidative stress.
4. Discussion

Normal cellular stress response is an important barrier to carcinogenesis. As one type of cellular stress, oxidative damage can arise from overproduction of ROS and deficient antioxidant and/or DNA repair systems [181]. Accumulated ROS can damage resident proteins, lipids, and DNA [186].

ATM has been shown as an important sensor of ROS in human cells [274], indicating that ATM plays a role in cellular redox defense to oxidative stress besides its role in DDR. In this study, our results was showing that down-regulation of HtrA1 in MCF10A cells activated the DNA damage detector, ATM, even before the hydrogen peroxide treatment, suggesting that HtrA1 might be one of the important factors for maintaining cellular redox homeostasis.

Furthermore, the acute oxidative stress treatment (hydrogen peroxide) generated DNA damage in MCF10A-based various cell lines. However, HtrA1 knockdown cells showed more severe DNA damage, which indicated by γH2AX foci, whereas the HtrA1 overexpression cell prevented the formation of γH2AX foci. This result suggests that HtrA1 could be an important ATM-network gene in response to DDR, as well as its role in redox defense.

We have also demonstrated that after the hydrogen peroxide treatment, ATM, CHK1, and CHK2 were all activated in all cells except in the HtrA1 overexpressing MCF10A/HtrA1 cell line, which did not show the phosphorylation of CHK1, suggesting that HtrA1 might be involved in the CHK1-directed cell cycle arrest. Therefore, a direction for future study is its role in the cell cycle arrest. For instance, we could further
check the phosphorylation status of downstream factors of CHK1, such as p53, CDC25, and even further, the CDC25 downstream effectors, CDK2 and CDK1, to pinpoint the cell cycle arrest phases, which may be affected by overexpression of HtrA1 in the MCF10A cell line. And another avenue to explore are the DNA repair enzymes showing significant changes in our gene array results.

Cancer cells are believed to have more endogenous DNA damage than non-cancer cells. In order to survive in such a genotoxic environment, cancer cells must have developed new ways to adapt to the stress, which may eventually result in resistance to chemotherapies. Therefore, the aberrations of DDR genes in cancer cells may not only facilitate the initiation and progression of cancer but may also cause the cells’ resistance to many conventional cancer treatments. In this study we show that HtrA1 expression could protect non-tumorigenic breast cells from acute ROS-induced DNA damage, whereas down-regulation of HtrA1 in the same setting predisposed cells to DNA damage. Therefore, the lower levels of HtrA1 expression associated chemoresistance might also relate to the important effects in DNA damage response, as well as to the EMT.

Therefore, it is very important to investigate deeply and precisely on HtrA1’s role in chronic oxidative stress and re-introducing/reactivating it in breast cancer cells to develop this potential therapeutic approach.
Chapter 7

Thesis Discussion

1. Thesis Summary

In this report, we demonstrated that HtrA1 gene was down-regulated in all of the breast cancer cell lines examined compared with non-tumorigenic breast epithelial cells and HtrA1 expression silencing was largely due to epigenetic modifications, including at least promoter DNA hypermethylation and histone deacetylation, and post-transcriptional mechanisms. Next, we created a series of MCF10A-derived cell lines to examine the effects of HtrA1 overexpression or knockdown. Notably, the decreased expression of HtrA1 stimulated cell growth and strongly correlated to the EMT phenotypes, encompassing down-regulation of epithelial cell biomarkers (E-Cad and cytokeratins); increased expression of mesenchymal cell markers (vimentin and N-Cad); decreased expression of miR200 family genes; and increased cell migration and invasion abilities. Finally, we found that HtrA1 overexpression could protect cells from acute oxidative stress-induced DNA DSBs, while HtrA1 knockdown activated the DNA damage sensor, ATM, and resulted in more severe DNA damage following acute oxidative stress. Taken together, the evidence lends support to the idea that HtrA1 may function as a tumor suppressor in human breast epithelial cancer. The schematic summary of this study is in Figure 7-1.
Dramatically decreased in breast cancer cells, involving epigenetic silencing mechanisms (Chapter 3)

?? HtrA1 Protein targets ??

Preventing EMT (Chapter 5) ?? Protection from ROS-induced DNA damage (Chapter 6)

Our results suggest a position of HtrA1 in the EMT induction network, in which it might be interacting with EGFR, up-stream of miR200s, and either downstream or parallel to TGFβ1 (Figure 7-2). In the HtrA1/EGFR/AKT pathway, when HtrA1 is down-regulated EGFR will be released from the HtrA1/EGFR interaction, thus to form EGFR dimer and activate its downstream signaling pathways, in which, AKT is one of the downstream transducers to be phosphorylated and activated. Then the activated AKTs could activate many effectors, such as GSK-3, MAP3K5, as well as members of the forkhead transcription factor family, to play important roles in the EMT. In the HtrA1/miR200s pathway, HtrA1 is a positive regulator of miR200s since down-regulation of HtrA1 in MCF10A cells dramatically decreased all five miR200 member genes in our miR array results. Down-regulated miR200s have been shown to promote EMT through the
transcription factor ZEB1, SIP1, and PTP-Pez, which is a TGFβ signaling inducer [118, 124]. However, we did not observe a direct correlation between HtrA1’s level vs TGFβ1’s level from cell culture medium in HtrA1 transfected cell lines, which suggested a possible parallel position of HtrA1 in the EMT pathways.

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**Figure 7-2: The hypothetical position of HtrA1 in EMT network.** Our results suggest that HtrA1 might play a role in EMT through interacting with EGFR and regulating microRNA200s. The possible relationship between HtrA1 and TGFβ1 is that HtrA1 is in an either parallel or downstream position of TGFβ1.

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### 2. Future Directions

We have shown our novel findings of HtrA1’s functions in this study (Figure 7-1). However, as a proteolytic enzyme, HtrA1 can have a variety of digestion substrates, and it may play its anti-tumor functions through interacting with all those factors.
Therefore, to search for its potential binding partners, we could perform the pull-down assay, which is an affinity approach for studying protein-protein interactions. Unlike IP or Co-IP, pull-down assay does not involve specific antibodies. It requires a purified and tagged HtrA1 protein as the bait to ‘pull-down’ the binding partners. The bait protein is created through cloning and expression of a fusion N-terminal 6His tag protein and add 5Glycine and 1 Cysleine in the C-terminal. After expression and purification, this protein can be attached onto the glass beads surface. The bait protein on glass beads is then incubated with a protein solution expressing protein(s) that may bind to the bait. These protein(s) can then be eluted and sequenced.

Human HtrA1 gene has 9 exons, which encodes 458 amino acids. From N-terminus to C-terminus it contains a signal peptide, insulin growth factor binding domain, Kazal protease inhibitor domain, trypsin-like protease domain, and a PDZ domain [15]. Which region(s) are crucial to its tumor suppressor functions and whether the anti-tumor properties are depending on its protease activity? Those could be a near future plan for HtrA1’s functioning study.

Each domain of the HtrA1 peptide can be constructed as a deletion mutation using appropriate restriction enzyme digestion. To examine whether the serine protease activity is necessary for it’s tumor-suppressing function, the serine at amino acid position 328 can be substituted with alanine by PCR mutagenesis. This S328A mutant exhibited no protease activity [30]. The series of HtrA1 mutant constructs can then be applied to the proposal of the inducible overexpression of HtrA1 in breast cancer cell lines mentioned in Chapter 4 discussion. And those corresponding HtrA1 mutant proteins can be used as the bait protein in glass beads-binding assay.
Cancer cell lines represent clonal outgrowths that have survived the selection pressures of in vitro culture and, as such, do not fully represent clinical cancers growing in situ. Thus, we have used a series of human tissue samples for the detection of oxidative stress, HtrA1, and EMT marker genes (Figure 7-3). Sample examples were coming from two reduction mammoplastics and one DCIS patient. In normal breast tissues, higher level of OxS, which is indicated by 4HNE staining, corresponds with higher levels of vimentin expression, and in that same sample, HtrA1 expression level is also high. Second, comparing the DCIS sample to normal ones, OxS level in this sample is higher and the vimentin expression is significantly increased surrounding the DCIS, whereas HtrA1 expression level is very low.

These results are supportive of a tumor suppressor role of HtrA1 in breast carcinogenesis. Our hypothesis is that in the normal stage cells, HtrA1 is acting to prevent DNA damage. HtrA1 expression level increases with increasing cellular stresses to prevent the EMT. However, after the cells are transformed, HtrA1 is silenced, and the EMT can occur, and loss of HtrA1 expression may actually be a prerequisite for transformation.

Characterization of all of these marker genes in human tissue samples is not an easy task. Sample size is the first challenge to get enough data to draw conclusions. Second, the specificity of all the antibodies is another issue that cannot be neglected.
As an alternative method to tissue immunostaining, laser Capture Microdissection (LCM) might have advantages. LCM facilitates removal of individual cells (or groups of cells) from histological specimens, thereby avoiding problems of tissue heterogeneity.
The isolated cells can be processed for downstream DNA, RNA, and protein experiments.

The definitive model to study the functions of HtrA1 gene in breast carcinogenesis will be a gene knockout mouse model, presumably combined with a chronic oxidative stress regimen. For example, the MCF10A cell line has been reported malignantly transformed into a basal-like cancer phenotype after continuous exposure to low-level cadmium for 40 weeks [213]. For creating this mouse model, we might want to use the Cre-loxP gene targeting system with breast tissue specific promoter to avoid the possible embryonic lethality.

So, the important aim of future research will be to define the precise stage of loss of HtrA1 in breast carcinogenesis.

3. Conclusion

Here, we show that HtrA1 gene expression was dramatically decreased, and even lost in human breast cancer cells compared with normal ones, and this decrease of HtrA1 might trigger the EMT and result in a predisposition to DNA damage.

The molecular mechanisms of proteolytic enzymes involved in anti-tumor functions may be very diverse. Because they have a variety of digestion substrates including growth factors, growth factor receptors, precursors of active peptides, pro-apoptotic ligands, intracellular and extracellular components, cytokines and other
proteases, etc [2]. HtrA1 could potentially play its tumor suppressor role through all these mechanisms.

Although we have not yet defined whether the silencing of HtrA1 gene in breast cancer cells is a driver or a passenger mutation, it nevertheless could represent a promising new marker, and a potential target in this disease.
References


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