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The Role of p62/IMP2 in Breast Cancer Progression

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THE ROLE OF p62/IMP2 IN BREAST CANCER PROGRESSION

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Abstract

Breast cancer is one of the most common types of cancer in women. In most patients with breast cancer, it is not the primary tumor that leads to death, but rather metastatic tumors. Once breast cancer spreads to other organs in the body, the disease becomes almost incurable. It is estimated that about 20% to 50% of patients diagnosed with primary breast cancer will develop metastatic disease. Considering this, it is important to understand the molecular and cellular mechanisms behind metastatic breast cancer and to identify new proteins that regulate the metastatic process. These proteins may be used as targets for possible therapeutic intervention. This study focuses on one such protein: p62/IMP2. We found that p62/IMP2, a member of IGF2 mRNA-binding protein (IMP) family, may play an important role in breast cancer metastasis.

In our study, the clinical data demonstrated that p62/IMP2 is highly associated with breast cancer. The overexpression of p62/IMP2 can be observed in breast cancer tissues, and a high frequency of its autoantibody can be detected in sera from patients with breast cancer. In our in vitro study, we found that overexpression of p62/IMP2 in breast cancer cell may promote breast cancer metastasis by increasing cell migration and reducing cell adhesion. In the study with the RT Profiler qPCR Array, we noticed that most changed genes with the overexpression of p62/IMP2 play important roles relating to cancer metastasis, such as cell migration and cell adhesion. Among the changed genes, CTGF was first identified as a novel target of p62/IMP2. In summary, p62/IMP2 may be an important regulator in breast cancer metastasis by controlling a group of adhesive molecules.

In future studies, we will focus on two points. 1) To confirm the results from in vitro study by using the in vivo experiments. In the in vivo study, we will investigate if p62/IMP2 really promotes breast cancer metastasis. 2) CTGF and p62/IMP2 expression in the breast cancer

cells will be detected and the staining of CTGF and p62/IMP2 will be performed in tumor tissues. The completion of this project is expected to expand our knowledge regarding the function of p62/IMP2 in breast cancer metastasis and further improve our understanding of the molecular process in the transition from primary breast cancer to metastatic breast cancer.

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Chapter 1: Introduction

1.1 Human breast cancer

Breast cancer is a type of cancer that develops from breast tissue and cells. There are more than 18 subtypes of breast cancer and they vary based on the site of tumor's origin and whether the tumor is in situ or invasive, or in the reproductive status [1]. The most common type of breast cancer is ductal carcinomas, which develops from the lactiferous duct (milk duct). Another common type is lobular carcinomas, a cancer that begins in the lobules. Based on a number of biological markers (proteins found in cells that have been associated with mechanisms tied to breast cancer), a newer set of breast cancer classifications has been established: basal, HER-2 over-expression, luminal A, luminal B, and unclassified [2]. These new classifications offer a better understanding the mechanisms behind breast cancer and provide newer therapeutic targets.

As research advanced and developments in diagnosis and therapeutic strategies developed, female breast cancer rates have gradually decreased since 2000. However, breast cancer is still one of the major causes of cancer-related death for women throughout the world. It is estimated that one in eight women born today will be diagnosed with breast cancer during their life.

The etiology of breast cancer is far from well-known. It is generally believed that both the combination of environmental factors and genetic predisposition contribute to the development of this disease. The environmental factors include obesity, alcohol consumption, estrogen exposure, radiation exposure, and aging [3, 4]. However, how these environmental factors increase the risk for breast cancer is still largely unknown. A genetic pre-disposition to breast cancer is estimated to cause only about 5%-10% of breast cancer cases. These well-known mutations include BRCA1, BRCA2, p53, PTEN, STK11, CHEK2, ATM, BRIP1, and PALB2

[5]. Among them, the mutations in the BRCA1 or BRCA2 tumor suppressor genes are the most important contributors to hereditary breast cancer. The BRCA1 and BRCA2 proteins are involved in various cellular processes. It has been demonstrated that BRCA1 and BRCA2 regulate some genes involved in DNA repair, the cell cycle, and apoptosis [6]. If the genes are mutated, the mutated BRCA1 and BRCA2 cannot properly repair damaged DNA, which increases the risk of breast cancer. However, there is still much to figure out in understanding the mechanisms, preventions and possible therapeutics for breast cancer.

1.2 Breast cancer metastasis

The major cause of the death in breast cancer is not caused by the primary tumor but rather by the metastatic disease. It is estimated that about 20% to 50% of patients diagnosed with primary breast cancer will develop metastatic disease [7]. Once breast cancer spreads to other parts of the body, the disease becomes almost incurable [8]. Therefore, breast cancer metastasis is a significant challenge that cancer researchers face [7]. To overcome this challenge, we must understand how breast cancer metastasis happens and how the mechanisms behind it operate.

Breast cancer metastasis is just like other cancer metastasis in the sense that the process consists of a series of successive steps. The invasion-metastasis cascade (Fig.1.) starts with the primary tumor invasion into adjacent normal tissues such as the stroma [9]. Cancer cells from the primary site then breach the basement membrane and penetrate the blood vessels, which releases them into the bloodstream. Within the circulatory system, cancer cells interact with other blood cells such as platelets to form cell lumps. These lumps can become stuck in blood capillaries when they travel through the body. After being trapped, cancer cells will subsequently extravasate and settle down in distant organs [10]. Then the secondary tumor deposits occur and the same process may be repeated, sometimes more rapidly.

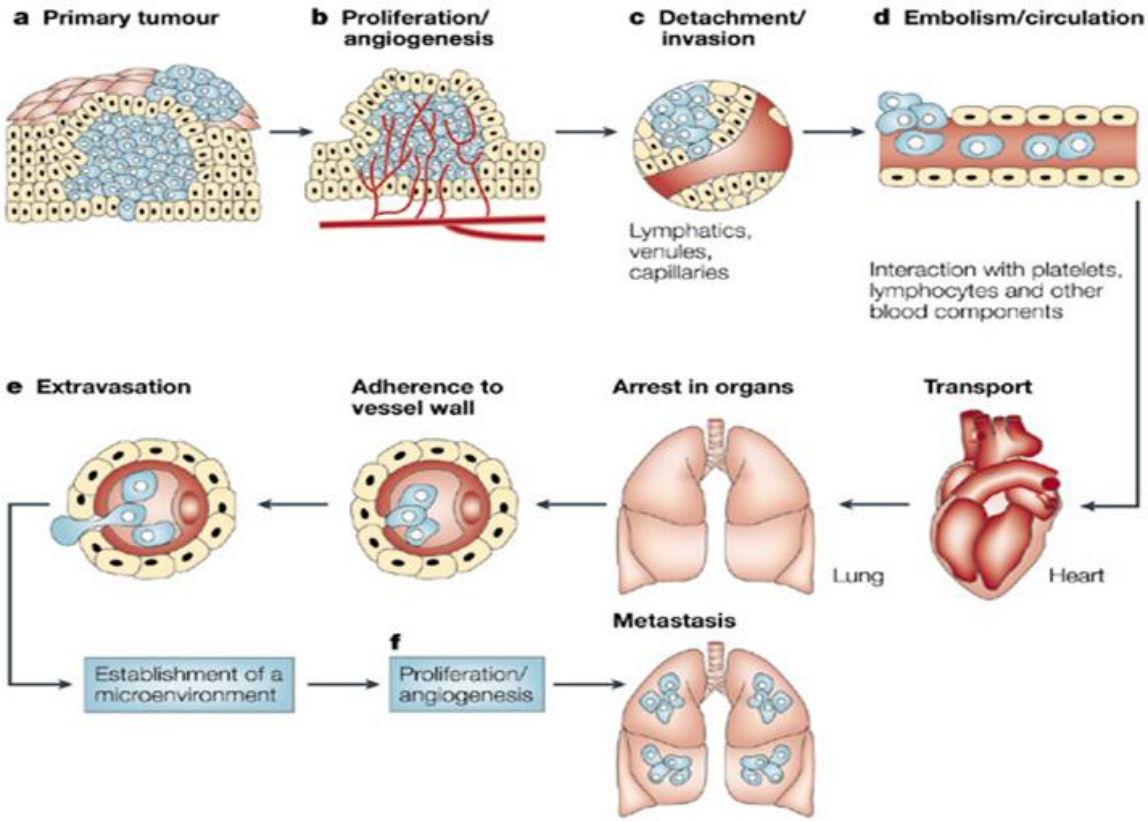


Figure 1: The invasion-metastasis cascade. Cancer cells grow in the primary site, then invade the local tissues and eventually go into the circulation. These cancer cells travel to the distant parts of our body, and settle down and grow at the secondary sites.

In terms of breast cancer in humans, the common sites of metastasis include bones, liver, lungs, and the brain [11]. Furthermore, cancer metastasis involves not only migration of cancer cells but also interaction between cancer cells and their surrounding cells. Cancer cells will induce fibroblast nearby to secrete stromelysin-3, which can break down the basement membrane. Cancer cells also induce macrophages surrounding them and secrete EGF to support them [12].

1.2.1 The role of epithelial-mesenchymal transition in breast cancer metastasis

Epithelial-mesenchymal transition (EMT) is a process through which epithelial cells lose the adherent tight junctions that keep them in contact with their neighbors and gain migratory properties to become mesenchymal cells (Table 1) [13]. Cancer cells at the edge of a primary

tumor undergo an EMT as they invade into the stroma and migrate to blood vessels. They must shed many of their epithelial phenotypes and detach from epithelial sheets to become mesenchymal. EMT causes cancer cells to invade locally, intravasate, and subsequently extravasate into a distant tissue. Once cancer cells grow in distant sites, these cells will regain an epithelial phenotype via a mesenchymal-epithelial transition (MET) [14].

Table 1: Changes associated with the epithelial-mesenchymal transition

Loss of	Acquisition of
Cytokeratin (intermediate filament) expression	Fibroblast-like shape
Epithelial adherents junction protein (E-cadherin)	Motility
Epithelial cell polarity	Invasiveness
	Mesenchymal gene junction protein (N-cadherin)
	Protease secretion
	Vimentin expression
	Fibronectin secretion
	PDGF receptor expression

Epithelial-mesenchymal transition is often induced by stromal signals. Loss of E-cadherin is considered to be a fundamental event in EMT. Many signaling pathways (TGF-beta, FGF, EGF, HGF, Wnt/beta-catenin and Notch) and hypoxia may induce EMT. Wnt/beta-catenin signaling pathway is very important to breast cancer metastasis. Active Wnt/beta-catenin pathway correlates with poor prognosis in breast cancer patients. Wnt in breast cancer cells can induce the EMT regulator SNAIL and upregulates the mesenchymal marker, vimentin [13]. Consistently,SNAIL is highly expressed in both epithelial and endothelial cells of invasive breast cancer but not observed in normal breast cancer. Transforming growth factor-β (TGF-β) signaling is implicated as the critical promoter of EMT. The activated TGF-beta/Smad signaling can contribute the form of the breast cancer bone metastasis [15].

1.2.2 The role of cell adhesion in breast cancer metastasis

It has been demonstrated that the alternation of cell adhesion is very critical in the

metastasis process of (breast) cancer. The invasive tumor cells must experience the change of cell to cell adhesion and cell-matrix adhesion [16, 17].

The most important 'cell to cell adhesion' is the loss of E-cadherin and the gain of N-cadherin in the EMT process. The shutdown of E-cadherin enables breast cancer cells to break away from the neighbor cells, and the high level expression of N-cadherin allows breast cancer cells to obtain contact with various types of mesenchymal cells, such as fibroblasts and endothelial cells that reside in the stroma. In this way, breast cancer cells disseminate from the primary tumor and obtain invasive capabilities for metastasis. Therefore the process of metastasis is also known as a cadherin switch.

The adhesion of breast cancer cells to the ECM (cell-matrix adhesion) can be mediated through integrins. Integrins are transmembrane receptors and they can bind to the components in extracellular matrix including fibronectin, vitronectin, laminin or collagen. Integrins can affect cellular shapes and their movement by controlling bidirectional signaling (inside-out signaling and the out-inside signaling). Activated integrins can also recruit proteases such as metalloproteinases (MMP) to the invading edge of a tumor and this can promote ECM degradation for easy spread of cancer cells.

Similarly, cell adhesion is necessary for tumor cells in circulation to interact with platelets and leukocytes to form new lumps for extravasation [18]. Let us take the immunoglobulin gene superfamily (IgSF) as an example. IgSF is a large group of cell surface and soluble proteins, and many members in this family are associated with metastasis in various cancers, including breast cancer. It is reported that IgSF is implicated in cancer spread by regulating the adhesion between circulating tumor cells and capillary endothelium or immunocytes [19].

Therefore, we can conclude that the deregulation of cell adhesion can be found in the

whole process of cancer metastasis and many adhesive molecules are found to be involved in it.

1.3 The microenvironment of breast cancer metastasis: Extracellular matrix

A successful metastasis requires a local microenvironment or niches to support cancer cells to survive, colonize and expand [20]. The major component of the niche is the extracellular matrix (ECM). The increased or reduced adhesion between cancer cells and extracellular matrix is in fact a result of the talking of cancer cells and ECM (a local microenvironment). The major contribution of this talking is to promote cancer metastasis, although there is evidence that talking also supports the growth of cells [16].

First, the angiogenesis in tumors depends on the stimulation of adhesion molecules. To keep growth and increase in size, cancer cells must consume more oxygen and nutrients and remove waste products at the same time. The formation of new blood vessels can supply more energy for cell growth [21]. More importantly, these blood vessels also serve as a way for systemic metastasis. It has been reported that soluble members of adhesion molecules are able to stimulate angiogenesis. Secondly, it is generally believed that cancer stem cells possess characteristics of both stem cells and cancer cells and hold the metastatic potential. Some evidence suggests that ECM is a key component of the cancer stem cell niche necessary for cells to acquire or maintain stem cell properties [22]. Consider how tenascin C, one of the ECM components, can modulate FGF2 signaling which is essential for neural stem cell biology. On the other hand, the loss of ECM contact reduces the number of stem cells.

In breast tissue, proteins of ECM not only work as a structural scaffold to maintain tissue integrity but also determine cell fate. ECM provides an adhesive surface for cells to bind growth factors and cytokine. The molecular signaling can be passed between cell and ECM in this way [23]. In breast cancer an increase in the deposition of fibrotic stomal matrix such as collagen,

elastin, vitronectin, and matricellular proteins can be observed. The important ECM components in breast cancer progression include hyluronan, matrix metalloproteinases, tenascin, and thrombospondin. Hyaluronan (HA) is a highly abundant oligosaccharide and forms a significant part of ECM. Hyaluronan is produced by Hyaluronan synthase (HAS). HAS can participate in all of the stages of cancer metastasis. By producing anti-adhesive HA, HAS allows tumor cells to release from the primary tumor mass. In animal models, expression of HAS is reported to support mammary tumor invasion and metastasis [24]. In contrast, the inhibition of HAS leads to reduced cancer progression.

Matrix metalloproteinsases is a sort of ECM modifying enzymes related with metastatic breast cancer. The overexpression of MMPs is helpful to break basement membrane partially by degrading collagen [25]. Tenascin is a member in the matricellular family and forms a critical constituent of provisional matrix of wounds. In breast cancer it is highly up-regulated. Tenascin C can also modulate FGF2 signaling, which is critical for neural stem cell biology. THBS1 is a component of platelets, and this protein has double roles in cancer progression. On one hand, THBS1 has been shown to represses growth of primary tumors. On the other hand, THBS1 is an effective inducer of metastasis [26]. It is known that THBS1 is able to promote lung metastasis and breast cancer metastasis, and its expression is associated with poor metastasis free survival [27]. In conclusion, breast cancer metastasis results from interaction of cancer cells and the microenvironment.

1.4 IGF2 mRNA binding proteins family

The family of insulin-like growth factor 2 mRNA binding proteins (IMP family) was first identified as a family of fetal RNA-binding proteins that exhibits high affinity to the IGF2 in 1999 [28]. This family has three members: IMP1 (63KD), p62/IMP2 (66KD), and IMP3 (64KD).

The expression of IMP family is biphasic, with an early expression in the oocyte and in the zygote, followed by a sharp peak in the expression from embryonic day 10.5 to 12.5 before a decline towards the birth of a mouse [29]. Compared with the high expression in the embryo, the expression of IMP family members is almost turned off in adult organs, except in reproductive tissues. However, in various tumors the overexpression of IMPs is induced. Therefore IMPs are viewed as oncofetal proteins [30].

Three members in the IMP family share similar protein structures in mammals. The sequence identity between them ranges from 60% to 70% and the similarity ranges from 80% to 85%. All

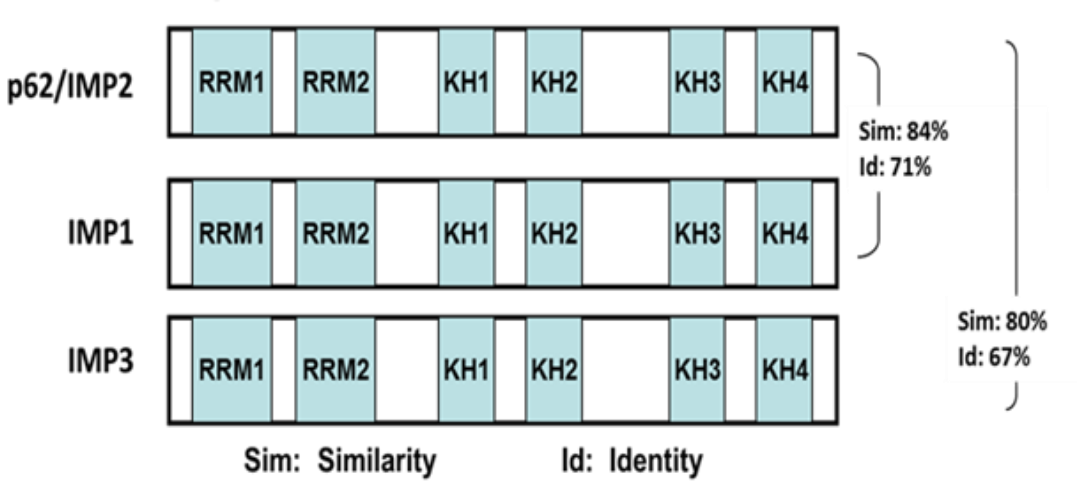


Figure 2: The comparison of three members' structure in IMPs. This figure showed that the similarity between p62/IMP2 and related RRM-KH proteins IMP1 and IMP3.

members have six characteristic RNA-binding domains, including two N-terminal RNA recognition motifs (RRMs) and four C-terminal heterogeneous nuclear ribonucleoprotein (hnRNP) K-homology (KH) domains (Fig.2.). The structure similarity further explains why these proteins share similar biological functions. As to the RRM domains, they are responsible for protein-protein interaction, protein dimerization, and the stabilization of IMPs-RNA complexes. As for KH domains, they are related with RNA-binding, granular RNP assembly, and RNA

localization [31, 29, 32, 33]. All three members are mainly cytoplasmic. In the cytoplasm, they form large RNP granules dispersed in the whole cytoplasm, around the nucleus, or in cellular protrusions. Additionally, they also have two nuclear export signals, which imply that they bind to their target mRNAs in the nucleus and export their target mRNAs from the nucleus to the cytoplasm [29].

Although IMPs are named as IGF2 mRNA-binding proteins, it has been known that they can bind to many different target transcripts in different cellular contexts, and most of targets are highly associated with tumor progression such as c-myc [34], beta-actin [35, 36], H19 [37], CD44 [38], MDR1 [39], RAS [40, 41] and many others (Table 2). Therefore IMPs are considered as valuable targets for cancer treatment. Within the IMP family, IMP1 (also named IGF2BP1, CRD-BP, VICKZ1, ZBP-1) has been extensively investigated. IMP1 can direct the localization of beta-actin mRNA to the leading edge of fibroblasts and increase cell motility in cancer cells [31, 42]. The IMP1 gene is amplified in breast cancer and its expression has been significantly associated with the absence of estrogen receptors [43]. Tissue specific induced expression of IMP1 in transgenic mice promotes tumor formation [44]. Recent work on IMP1 protein structure has paved the way towards future drug design, possibly through fragment-based screening or virtual ligand screening to inhibit binding of substrates such as the MYC or IGF2 mRNAs [45, 46]. p62/IMP2 has been extensively validated as a potential biomarker for early cancers diagnosis [47, 48]. IMP3 is reported to be related with tumor aggressiveness and its expression is a good predictor of prognosis in individual patients.

Table 2: Most important targets of IMPs

Proteins	Target mRNAs	Function of target mRNAs
IMP1,2,3	IGF-Ⅱ	Growth-regulating, insulin-like, mitogenic activities
IMP1,2,3	H19	Oncogene. Invasion, migration and angiogenesis
IMP1,2,3	c-myc	Cell proliferation, growth and apoptosis
IMP1,3	CD44	Cell–cell interactions, cell adhesion and migration
IMP1	β-actin	The polarity of cell for directional movement
IMP1	Tau	Microtubules stability
IMP2	MURF-3	Microtubules stability

1.5 IMP2 gene, IMP2 protein and the regulation of IMP2

It is known that three isoforms of IMP2 are expressed in cancer cell lines. The longest one is IMP2 with 66KD [28]. p62 (Acc. No.: NM001007225.1) is an isoform that extron 10 is truncated from the IMP2 gene, which leads to 43 amino acids being omitted between KH2 domain and KH3 domain [49]. The shortest isoform is p58, resulting from leaky scanning of IMP2 [50]. p58 lacks the first RRM domain in the N-terminal, so the binding affinity and specificity to target mRNAs and protein partners is affected [51].

The IMP2 gene is located on 3q27.2. It is encoded by 16 extrons and 15 introns [29]. There is an AT-rich regulatory region in the first intron; HMGA2 can bind with this AT-rich region to regulate IMP2 [52]. Interestingly, HMGA2 was also an oncofetal protein [53] so it has a similar expression pattern to p62/IMP2. More importantly, HMGA2 is has recently been reported to be a driver in cancer metastasis by inducing the epithelial-mesenchynal transition (EMT) [54]. Therefore p62/IMP2 also has a potential role in promoting cancer metastasis as an important target of HMGA2. The second intron of IMP2 gene is the largest intron by far, in which a single nucleotide polymorphism is reported to be associated with type 2 diabetes [55, 56]. p62/IMP2 can bind different mRNAs to direct the fate of cells, and the binding is controlled by its phosphorylation [55, 56]. It has been suggested that two serine sites (Ser162/Ser164) in the

N-terminal linker region of p62/IMP2 (between RRM2 domain and KH1 domain) can be phosphorylated by mTOR, which will promote the binding with IGF2 and increase IGF2 protein synthesis [57].

1.6 p62/IMP2 and cancer

1.6.1 p62/IMP2 is a tumor-associated antigen and its autoantibody is viewed as a potential biomarker in early detection of cancer

Tumor-associated antigens (TAAs) are antigens that are highly correlated with tumor cells. Many tumor cells produce tumor-associated antigens, which may be released in the bloodstream or remain on the cell surface. TAAs have been identified in most of human cancers including prostate cancer, malignant melanoma, and breast carcinoma [58]. The origin for TAAs may be mutated proteins in cancer or abnormally high level of proteins in cancer that have a low expression in normal cells [59]. These TAAs can be sensed by the human immune system as foreign antigens and stimulate the immune system to produce the corresponding autoantibody. Because these autoantibodies are stable in patients' sera and easily detectable by ELISA [60], they are viewed as a sort of biomarker in the early detection of cancer.

p62/IMP2 was first identified as a tumor-associated antigen known as p62 in hephatocellular carcinoma by immunoscreening cDNA library from sera of patients with liver cancer. This cytoplasmic protein p62 is an mRNA binding protein and belong to IMP family binding to insulin-like growth factor 2 mRNA. The frequency of the autoantibody against p62/IMP2 in sera from cancer patients is significantly higher than it in sera from healthy individuals. The detectable antibody was present in 21.1% of patients with HCC from China but not in the precursor diseases of HCC. In a study involving ovarian cancer, the frequency of

autoantibody to p62/IMP2 was 29.4% in patients and 1.1% in healthy individuals [61]. Of 83 patients with digestive canal tumors, 32 showed the positive serum autoantibody to p62 (38.55%) and 28 had metastasized (33.7 %) [62]. In prostate cancer, the prevalence of serum autoantibody to p62/IMP2 was 25.2% in patients [63]. In esophageal cancer, the autoantibody of p62/IMP2 was detected in 16.8% of 119 esophageal cancer patients. In addition, a relatively higher frequency of autoantibody response to IMP2/p62 was observed in patients with colon cancer (23.4%, 15/64) compared to patients with colonic adenomas (4.8%, 2/42) and normal individuals (2.9%, 1/34) [64]. This data suggested that p62/IMP2 and its autoantibody are closely associated with cancer development. More importantly, the significant rise of autoantibody of p62/IMP2 is also corresponding with the occurrence of cancers. As illustrated in Figure 3, four serum samples

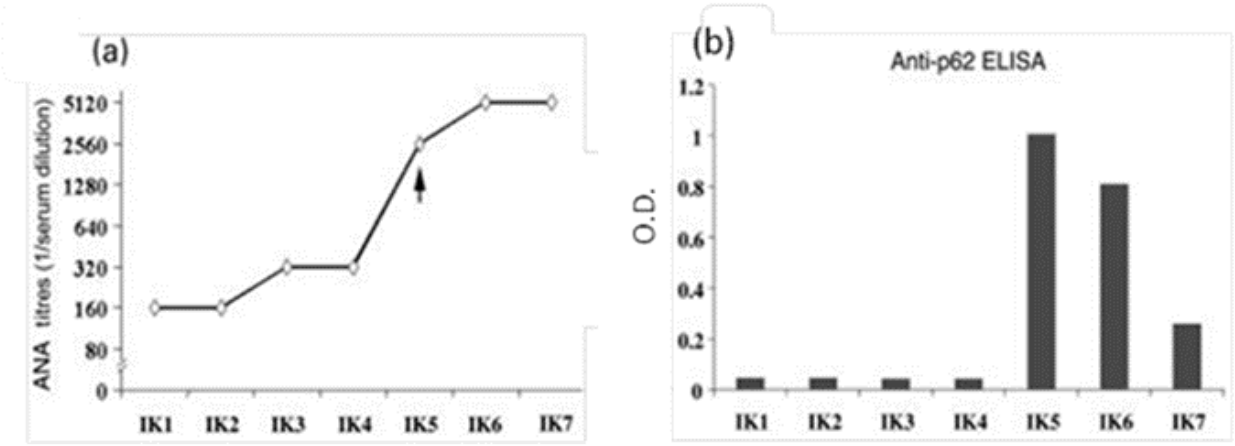


Figure 3: The appearance of autoantibody to p62/IMP2 coincides with the detection of HCC.

(IK1, IK2, IK3 and IK4) collected from one patient before the diagnosis of HCC had low ANA titers. When the diagnosis of HCC was made at time point IK5 (arrow), the ANA titer had increased significantly and persisted at a high titer for 6 months. From this case, we can see that the autoantibody of p62/IMP2 can be detected in the early stage of cancers, even one year before the onset of cancers [48]. Therefore, the autoantibody of p62/IMP2 may be an ideal biomarker in

the early detection of cancer. In the following study, more sera with serial years from patients should be collected to evaluate if it is a real biomarker in the early detection.

Studies demonstrated that tumor diameter is related to 5-year survival rates after surgery. Therefore, early detection of cancer is very important for promoting patients' survival rates. The detection of the autoantibody with ELISA is noninvasive and easily acceptable in clinical settings [60]. Based on this, p62/IMP2 is a valuable potential biomarker in the early diagnosis.

1.6.2 The role of p62/IMP2 in cancer

To support cell proliferation and stem cells survival, Some reports suggested that p62/IMP2 can promote cell survival because it can bind to a group of mRNAs such as c-myc, specificity protein 1 (Sp1), and Insulin-like growth factor 1 receptor (IGF1r) [65, 40]. Embryonal rhabdomyosarcoma (ERMS) growth and survival relies on the HMGA2-p62/IMP2–NRAS signaling pathway, and myoblast proliferation and myogenesis are also regulated by HMGA2-p62/IMP2 [66]. Other reports suggested that p62/IMP2 controls energy production pathways that are important for preserving cancer stem cells. In glioblastoma, p62/IMP2 regulates oxidative phosphorylation (OXPHOS) in primary glioblastoma sphere culture (gliomaspheres). Depletion of p62/IMP2 inhibits high oxygen consumption in gliomaspheres. It has been demonstrated that p62/IMP2 can bind several mRNAs such as the mRNA of COX7b, which encode mitochondrial respiratory chain complex subunits and that p62/IMP2 interacts with NADH dehydrogenase (ubiquinone) iron-sulfur protein 3 (NDUFS3) [67].

To promote cell migration: important cluster targets of p62/IMP2 consist of proteins (LIMS2, TRIM54, and LAMB2) involved in cell migration, cell adhesion, and cytoskeleton remodeling. LIMS2 is an LIM domain protein that binds to and partially dissociates the focal adhesion complexes [68]. TRIM54 is a ring finger protein specifically expressed in skeletal

muscle. In p62/IMP2 knockdown RMS cells (RD), LIMS2 is found to be upregulated and TRIM54 is found to be down-regulated for reducing cell migration, which leads to aggregation of focal adhesion structures [68]. Conversely, it means that p62/IMP2 expression can keep cell motility. Studies also provide evidence that p62/IMP2 targets LAMB2 mRNA to the actin cytoskeleton and regulates LAMB2 translation [69]. LAMB2 is a subunit of laminins, major extracellular glycoproteins in basement membranes. LAMB2 is thought to mediate the attachment, migration, and organization of cells into tissues during embryonic development by interacting with other extracellular matrix components. This data suggests that IMP2 can enhance the process of cell motility. The previous research on p62/IMP2 suggest that overexpression of p62/IMP2 plays an important role in the progression of cancer.

Chapter 2: Hypothesis and Aims

2.1 Specific Aim

Breast cancer is a complex and heterogeneous disease. The progression of it depends on both the alternation of cancer cells themselves and the deregulation of its surrounding microenvironment. Although our ability to diagnose and treat breast cancer is improving, breast cancer is still one of the most common human malignancies in women worldwide. Therefore, it is necessary to identify new proteins that regulate breast cancer as such proteins may be future targets for possible therapeutic intervention.

IGF2 mRNA-binding Protein 2 (IMP2, also named as IGF2BP2 or p62) belongs to the IMP family of proteins that consist of three members: IMP1, IMP2, and IMP3. All the members of this family are oncofetal proteins. They were initially found as a group of IGF2 mRNA-binding Proteins in rhabdomysarcoma cells. Then, other important target mRNAs were discovered, including c-myc, beta-actin, H19, CD44, MDR1, and RAS. Interestingly, most of the target mRNAs are related to progression of cancer.

It has been reported that the expression of p62/IMP2 is associated with several tumor types. Recently, researchers have suggested that p62/IMP2 can control the expression of proteins related to cell survival, growth, and movement. However, the mechanisms underlying this phenomenon remain largely elusive. In this proposal, we will focus on p62/IMP2 in breast cancer and hypothesize that it plays an important role in the progression of breast cancer progression **Thus, the goal of this proposal is to explore the role of p62/IMP2 within the progression of breast cancer via both in vitro and in vivo studies, as well as identify some novel targets of p62/IMP2 or its related genes in breast cancer cells.** The completion of this proposal will expand our knowledge of p62/IMP2 function in breast cancer. We will focus on the following

aims:

Specific aim 1: The relevance of p62/IMP2 to human breast cancer.

In this specific aim, p62/IMP2 expression will be evaluated by immunohistochemistry on human breast cancer tissues. In addition, the frequency of its autoantibody will be evaluated by ELISA with sera from breast cancer patients. These studies will determine whether p62/IMP2 is associated with breast cancer or not.

Specific aim 2: The impact of p62/IMP2 on breast cancer progression.

To evaluate the role of p62/IMP2 in breast cancer, a panel of breast cancer cells will be screened. Then, we will implement transfection or shRNA approaches to alter the expression of p62/IMP2. The generated variants will be used to test the impact of p62/IMP2 on the cell behavior of breast cancer via a series of in vitro assay, and in vivo mouse model.

Specific aim 3: The mechanism of p62/IMP2 in breast cancer progression

Although several research articles have reported on the target mRNAs for p62/IMP2, they varied from different cancer cell lines. To identify novel targets of p62/IMP2, which is specific to breast cancer, we will first use qPCR arrays to exame those genes (candidates) whose expression are altered following the de novo expression of p62/IMP2 in breast cancer cells. Next, we will test if p62/IMP2 binds to mRNAs of the candidates via RNA immunoprecipation (RIP). The candidates whose mRNAs can be pulled down together with p62/IMP2 by RIP are targets, and other candidates are related genes of p62/IMP2.

2.2 Significance

Breast cancer can be categorized into several different subtypes on the basis of different biomarkers. Well-known breast cancer biomarkers include the estrogen receptor (ER), the progesterone receptor (PR) and the HER2 gene [70, 71]. However, more biomarkers are needed to further characterize different types of breast cancer. Since p62/IMP2 is a tumor-associated antigen, we hypothesize that we will find a significant percentage of clinical breast cancer tissues that overexpress p62/IMP2. Both immunohistochemistry analysis and ELISA analysis of sera will evaluate whether p62/IMP2 can be used as a biomarker for breast cancer.

The role of p62/IMP2 in cancer progression is still controversial, in part because of the lack of preclinical data. In our project, we will perform both in vivo and in vitro studies to examine the role of p62/IMP2 in breast cancer. The results from our in vitro study will provide evidence for a better understanding of breast cancer progression at the molecular and cellular level. Through the identification of p62/IMP2 novel targets in breast cancer cells, a series of novel genes essential in breast cancer progression may be identified in this way, which will contribute to our understanding of breast cancer at the molecular level. Results from our in vivo study will confirm the results from the in vitro study and make our data more solid and reliable. Also, the results of this proposal may provide targets that can be applied to future anti-metastatic drug design.

In conclusion, breast cancer still remains the second leading cause of cancer-related death in women (after lung cancer) [7]. In 2014, an estimated 232,670 new cases of invasive breast cancer were expected to be diagnosed, along with 62,570 new cases of non-invasive breast cancer (also known as carcinoma in situ). About 40,000 women were expected to die in 2014 from breast cancer, though there has been a decrease in death rates since 1995. The aim of this

project is to identify molecular targets for future therapies of breast cancer, which could ultimately lead to a decrease in breast cancer mortality. We hope that the accomplishments in our research will broaden our knowledge about breast cancer progression and provide theoretical basis for diagnosis and therapy.

Chapter 3: Materials and Methods

3.1. Cell lines and cell culture

Human breast cancer cells (MDA-MB-231, LM2-4, and MCF7) were cultured in Dulbecco's Modified Eagle Medium (GIBCO, Life Techologies, Grand Island, NY, USA). Human breast cancer cell line SKBr3 was maintained in McCoy's 5A medium (GIBCO, Life Techologies, Grand Island, NY, USA). The medium was supplemented with 10% fetal bovine serum (GIBCO, Life Technologies, Grand Island, NY, USA) and 100 units/ml penicillin plus 100 μg/ml streptomycin (Thermo Scientific, Waltham, MA, USA) at 37°C with 10% CO_2. Breast cancer cell lines (MDA-MB-231 and LM2-4) were a kind gift from Dr. Francia (UTEP, El Paso, TX).

3.2. Transfection

For transfection treatment, breast cancer cells were plated in 6-well plates and transfected using Lipofectamine2000 (Life Techologies, Grand Island, NY, USA) according to the manufacturers' instructions. To obtain stably transfected cell clones, cells with transient transfection were cultured in dishes with DMEM medium containing 1mg/ml G418 for 3 weeks. After the formation of cell clones, they were picked up using cloning cylinders (Corning, Tewksbury, MA, USA) and cultured in 96-well plates for growth. Cell lyses from each clone were collected for Western Blotting to determine which one are p62/IMP2 positive clones or p62/IMP2 negative clones.

3.3. Enzyme-linked immunosorbent assay (ELISA)

Polystyrene 96-well microtiter plates (Thermo scientific, Waltham, MA, USA) were coated overnight at 4℃ with purified recombinant p62/IMP2 at the final concentration of 0.5 μg/ml in phosphate-buffered saline (PBS). Then plates were blocked with 300μl post-coating solution for 2 hours. After washing away the post-coating solution, antigen-coated wells were incubated with 100μl human sera diluted at 1:100 for 2 hours. The antigen-antibody complex were further bound to HRP-conjugated goat anti-human IgG (Santa Cruz Biotechonolgy, Inc., Santa Cruz, CA, USA) and detected by the substrate 2, 2'-azino-bis-3-ethylbenzo-thiazoline-6-sulfonic acid (ABTS) (ABTS: Sigma-Aldrich, St. Louis, MO, USA). Optical density (OD) value at a wavelength of 405nm was monitored and collected for data analysis.

3.4. Immunohistochemistry

Multiple breast cancer tissue microarrays were purchased (US Biomax, Inc. MD, USA), Inc. There were total 104 paraffin-embedded breast cancer tissues and 14 normal breast tissues on slides. Briefly, after deparaffinization with xylene and rehydration with ethanol, antigen retrieval was performed by microwave heating methods. 2-3 drops of avidin block solution were applied to the slide for blocking endogenous biotin activity. After washing, 200μl p62/IMP2 antibody (ABCAM, Cambridge, MA, USA) with 1:100 dilutions was applied to cover every slide for incubation overnight at 4℃. Then the slides were incubated with Polyvalent Biotinylated Link as secondary antibody for 1 hour at room temperature. DAB reagent was used as detection and then was washed away immediately. At last, the slide was counterstained with hematoxylin, dehydrated with ethanol and stabilized with xylene and then processed for imaging. All IHC results were read blindly by two independent researchers and further confirmed by a

pathologist. A four-level scoring system (-, negative; +, weak expression level; ++, moderate expression level; +++, high expression level) was used to evaluate the staining intensity as previous study.

3.5. Cell Immunofluorescence

For cell immunofluorescence, cells were cultured into 8-chamber culture slides. Then cells on slides were fixed with 4% paraformaldehyde and incubated with 100 μl p62/IMP2 antibody with 1:500 overnight at 4°C. After washing, 100 μl FITC-conjugated goat anti-human IgG (1:100 dilutions) was used as secondary antibody. After washing again, mounting medium with DAPI (VECTOR Laboratories, Inc. Burlingame, CA, USA) was applied across the slides for nucleus staining and covering slides. Confocal fluorescence images were acquired with a laser scanning microscope (LSM 700; Zeiss, New York, NY).

3.6. Proliferation assay

Cells were seeded on to 96-well plates at $5*10^3$ per well, grown for 1 to 7 days. The growth of cells was determined by the sulforhodamine (SRB) assay. Briefly, the cells were fixed with 10% trichloracetic acid (TCA) for 1 hour at 4°C, rinsed five times with water, and air dried. The cells were then fixed with 0.4% SRB in acetic acid for 15 minutes. After washing, unbound dye was removed by washing 5 times with 1% acetic acid and plates are air dried. Bound staining was solubilized with 10Mm Tris Base. The colorimetric reading was carried out in a microplate autoreader at 515nm.

3.7. Wound healing assay

Cells were seeded in 6-well plates and cultured in DMEM medium with 2% FBS. After growing as a confluent cell monolayer, wound scratch was created by tips on cell monolayer. Detached cells were washed away. Scratch images of wound were photographed every 6 hours. The level of cell migration into the wound scratch was quantified as migration area at each time point with Image J software.

3.8. Cell-extracellular matrix adhesion assay

96-well plates were coated with 10ug/ml fibronectin or 10ug/ml type I collagen, and incubated overnight at 4°C. After washing, non-specific binding sites were blocked with 1% BSA in serum-free culture medium. The cells ($5*10^3$) were added to the coated 96-well plates and incubated at 37°C for 1 to 2 hours. Non-adherent cells were removed by washing with washing buffer (0.5% BSA in serum-free cell culture medium). Attached cells were stained by crystal violet (5mg/ml in 20% ethanol). After unbound dye was washed away, and bound staining was solubilized. The optical density was measured at 550 nm.

3.9. RNA isolation and quantitative real-time RT-PCR (qRT-PCR)

Total RNA from breast cancer cells was isolated by AllPrep DNA/RNA/Protein Mini Kit (QIAGEN, Valencia, CA, USA). RNA integrity was assessed by 0.7% agarose gel electrophoresis. RNA was reverse transcribed with GoScriptTM Reverse Tanscription System. To evaluate the relative level of p62/IMP2, and c-Myc, quantitative real-time PCR was

performed with MyiQ Single Color Real-time PCR Detection System. RT Profiler PCR Array kit was also performed in the same way.

3.10. RNA immunoprecipation

P62/IMP2-bound RIP was performed according to the RiboCluster Profiler RIP-Assay Kit (Medical & Biological Laboratories CO., LTD. Japan). Briefly, cell lysate was collected from p62/IMP2 positive cells, and then the whole complexes of p62/IMP-targets from cell lysate were pulled down with p62/IMP2 antibody. The target mRNAs were separated and isolated by solution from RIP-Assay Kit for the reverse transcription.

3.11. mRNA half-life analysis

p62/IMP2 positive cells and control cells (10^6) were seeded in to 6-well plates. After treated with 5ug/ml actinomycin D (Sigma), these cells were collected after every 30 minutes or every 2 hours. Total RNA were isolated and from cells collected from different time points. The amount of GAPDH, c-myc and CTGF mRNAs was quantified by Real-Time RT-PCR.

3.12. Cell proliferation assay

Cells were seeded to 96-well plates at $5*10^3$ per well, grown for 1 to 7 days. The growth of cells was determined by the sulforhodamine (SRB) assay. Briefly, the cells were fixed with 10% trichloracetic acid (TCA) for 1 hour at 4°C, rinsed five times with water, and air dried. The cells were then fixed with 0.4% SRB in acetic acid for 15 minutes. After washing, unbound dye was removed by washing 5 times with 1% acetic acid and plates are air dried. Bound staining

was solubilized with 10Mm Tris Base. The colorimetric reading was carried out in a microplate autoreader at 515nm.

3.13. Western blotting analysis

Protein from cell lysates were separated on 10% SDS-PAGE gels and wet transferred to nitrocellulose membrane (GE Healthcare Life Sciences, Pittsburgh, PA, USA) and then blocked for 1 hour at room temperature by 5% dry milk in TBS-T buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 0.1% Tween 20). Membranes were then incubated overnight at 4°C or 1 hour at room temperature with the respective primary antibodies: anti-p62/IMP2 (1:1000), anti-c-myc (1:1000), and anti-actin (1:1,000), (Santa Cruz Biotechonolgy, Inc., Santa Cruz, CA, USA). Then Anti-mouse or anti-rabbit secondary antibodies conjugated to horseradish peroxidase (Santa Cruz Biotechonolgy, Inc., Santa Cruz, CA, USA) were used. X-ray films were developed and results were visualized.

Chapter 4: p62/IMP2 is highly associated with human breast cancer

4.1 Overview

A family of recently indentified IGF 2 mRNAs binding proteins (IMP1, IMP2, and IMP3) is of particular interest in relation to tumorigenesis. This project focuses on one specific IMP family member, p62/IMP2. This proposal is organized into three parts to explore its role in breast cancer progression. The first part deals with the association between p62/IMP2 and breast cancer. The second part is the evaluation of the role of p62/IMP2 in breast cancer progression, and the third one focuses on the function of p62/IMP2. In this way, the clarification and validation of its role will allow for a better understanding of the molecular mechanisms of breast cancer and also provide possible targets for treatment. In chapter 4, we will focus on determining the association between p62/IMP2 and breast cancer.

4.2 Rationale, experimental design and alternative approach

***Specific aim 1*: The relevance of p62/IMP2 to human breast cancer progression.**

4.2.1 Rationale:

p62/IMP2 is considered to be involved in cancer because it is originally defined as a tumor-associated antigen as well as an oncofetal protein [30, 28]. A high frequency of autoantibody to p62/IMP2 can be observed in cancer patients' sera, and overexpression of it has been described in several tumors in recent reports, including liposarcomas [52], hepatocellular carcinomas [72], and endometrial adenocarcinomas [73]. Also, p62/IMP2 was reported to be expressed in some tumor cell lines (HepG2, SNU449, H1299, U-2OS, HT-29, PANC-1, and 1F6) [31]. However, there is limited research on the expression of p62/IMP2 in breast cancer, which contrasts with the extensive research on its homolog IMP1 [43, 44]. As a result we are interested in the relationship

between p62/IMP2 and breast cancer. The goal of specific aim 1 is to evaluate the expression of p62/IMP2 in human breast cancer tissues and the prevalence of its autoantibody in sera from breast cancer.

4.2.2 Experimental Design & Methods:

1) Analysis of p62/IMP2 expression in breast tumor tissues: we performed immunohistochemistry (IHC) to analyze p62/IMP2 expression in tumor tissues by using multiple breast tissue microarrays (which can provide a high throughput analysis of hundreds of tissue samples) so we can get a more accurate result. These tissue microarrays contained paraffin-embedded tissue specimens collected from patients and labeled with different grades ranging from Ⅰ-Ⅲ, metastasis or non-metastasis, and adjacent non-tumor tissues. Tissue microarrays were stained with antibody against p62/IMP2. The results from the IHC were analyzed by comparing normal and tumor tissues, non-metastatic and metastatic tissues, and tissues corresponding to different tumor stages.

2) Analysis of autoantibody of p62/IMP2 in sera from patients with breast cancer: ELISA was used to screen a group of sera from patients with breast cancer. Human sera included in the present study were obtained from the serum bank at the Cancer Autoimmunity and Epidemiology Research Laboratory at UTEP (University of Texas at El Paso). None of patients with breast cancer received treatment with any chemotherapy or radiotherapy. Sera were from normal individuals who had no obvious evidence of malignancy during annual health examinations. Recombinant p62/IMP2 protein was purified and coated in 96-well plates as described before.

4.2.3 Statistical approach:

Tissue microarrays that stained with p62/IMP2 antibody were scored as: 0(-) = no staining, 1(+) = low staining, 2(++) =moderate staining, 3(+++) = strong staining. Protein expression between tumor tissues and normal tissues was determined by using the Chi-square test (*: $P<0.01$, compared with normal group). The mean OD value of the group of patients' sera was compared using the Mann-Whitney U test. The frequency of antibody against P62/IMP2 in the group of patient sera was analyzed using the χ^2 test with Yate's correction.

4.2.4 Potential problems & alternative approaches:

We expect that the expression of p62/IMP2 is related to breast cancer and is highly expressed in advanced stages of breast cancer. Immunohistochemistry is regaded as a well-established method of evaluating protein expression. However, it depends on the use of an antibody with high specificity and sensitivity. Although we have developed an p62/IMP2-specific antibody in our lab and commercial antibodies of p62/IMP2 are available now, some false negative and positive results cannot be avoided entirely. One solution for this problem is to analyze substantial amount of samples to get correct clinical results. Another alternatie approach is to detect expression of p62/IMP2 via TissueScan Cancer Q-PCR analysis.

4.3 Results

4.3.1 p62/IMP2 is highly associated with breast cancer

To test the p62/IMP2 overexpression in breast cancer, we performed immunohistochemistry analysis of p62/IMP2 protein expression in human breast tissues using tissue microarrays. A total of 118 breast tissue cores were analyzed on slides, which contained 104 tumor tissue sections and 14 adjacent normal tissue sections. The data we obtained was summarized in Table 3, which

showed that a high expression of p62/IMP2 (69% 72/104) was seen in human breast tumor tissues and that a low expression of p62/IMP2 (2/14, corresponding to 14% cases) was seen in the adjacent normal tissues. We also tried to determine whether there was a correlation between p62/IMP2 expression and the different stages of breast cancer. However, our data did not support this notion (Table 4). Figure 4 showed 4 representative examples of the range of p62/IMP2 staining that were analyzed in this tissuearray study using a 4-level scoring system.

p62/IMP2 is also viewed as a tumor-associated antigen. The high frequency of autoantibodies to p62/IMP2 in sera from cancer patients strongly suggests that this protein is linked to cancer progression. To detect the prevalence of autoantibody against p62/IMP2 in human breast cancer, recombinant p62/IMP2 protein was used as coating antigen in ELISA to screen patients' sera with breast cancer and benign tumor, and sera from normal individuals. As shown in Table 5, analysis of sera from 216 patients with breast cancer (including sera from German, Chinese and Mexican patients) showed that the positive frequency of detectable autoantibody to p62/IMP2 was 29% (63/216). This was significantly higher than that observed in the sera from normal individuals and from patients with benign tumors (1%, 0%). From the distribution of optical density (OD) value of the three groups (Fig.5.), it was evident that most sera from patients with breast cancer were above the cutoff value (0.32), indicating that these sera were positive for p62/IMP2 autoantibody. In contrast, only one serum (OD: 0.54) from normal individuals was above the cutoff value, and no sera from patients with benign breast tumors was above the cutoff value.

This clinical data showed that the p62/IMP2 was over-expressed in breast cancer and the overexpressed p62/IMP2 in breast cancer can stimulate the immune system to produce its autoantibody. Therefore we can conclude that p62/IMP2 is highly associated with breast cancer.

Table 3: p62/IMP2 immunohistochemistry on normal and malignant human breast tissue

Type of Tissues	Positive staining			
	+++	++	+	total
Tumor Tissues(104)	10 (14%)	23 (32%)	39 (54%)	72/104 (69%)*
Normal Tissues (14)	0 (0%)	1 (7%)	1 (7%)	2/14 (14%)

Tissue microarrays stained with p62/IMP2 antibody were scored as: (-) = no staining, (+) = low staining, (++) =moderate staining, (+++) = strong staining. Protein between tumor tissues and normal tissues was determined using the Fisher's exact test. (*: $P<0.01$, compared with normal group)

Table 4: Immunohistochemical analysis of human breast tumors with stages using anti-p62/IMP2 antibody

Score	Positive staining			
	+++	++	+	total
Stage Ⅰ(7)	2(29%)	0(%)	3(43%)	5/7(71%)
Stage Ⅱ(49)	4(8%)	13(27%)	16(33%)	33/49(67%)
Stage Ⅲ(7)	2(29%)	4(57%)	1(14%)	7/7(100%)
Stage Ⅳ(1)	0	0	1	1/1(100%)

Scoring Criteria: Tissue microarray stained with antibody against p62/IMP2 were scored as: 0(-) = no staining, 1(+) = low staining, 2(++) =moderate staining, 3(+++) = strong staining.

Stage Ⅰ or well-differentiated: Cells appear normal and are not growing rapidly.

Stage Ⅱ or moderately-differentiated: Cells appear slightly different than normal.

Stage Ⅲ or poorly differentiated: Cells appear abnormal and tend to grow and spread more aggressively.

Stage Ⅳ or undifferentiated: (for certain tumors), features are not significantly distinguishing to make it look any different from undifferentiated cancers which occur in other organs.

Table 5: Frequency of autoantibody responses to p62/IMP2 protein

Group	Total	Positive (%)
Breast cancer	216	63 (29%)*#
Benign	34	0 (0%)
Normal	73	1 (1%)
Total	323	64

Cutoff value: Mean+3SD of normal group

Breast cancer group vs Normal group: * $p<0.01$

Breast cancer group vs Benign group: # $p<0.01$

Breast cancer: sera from patients with breast cancer

Benign: sera from patients with benign tumor in breast

Normal: sera from normal individuals

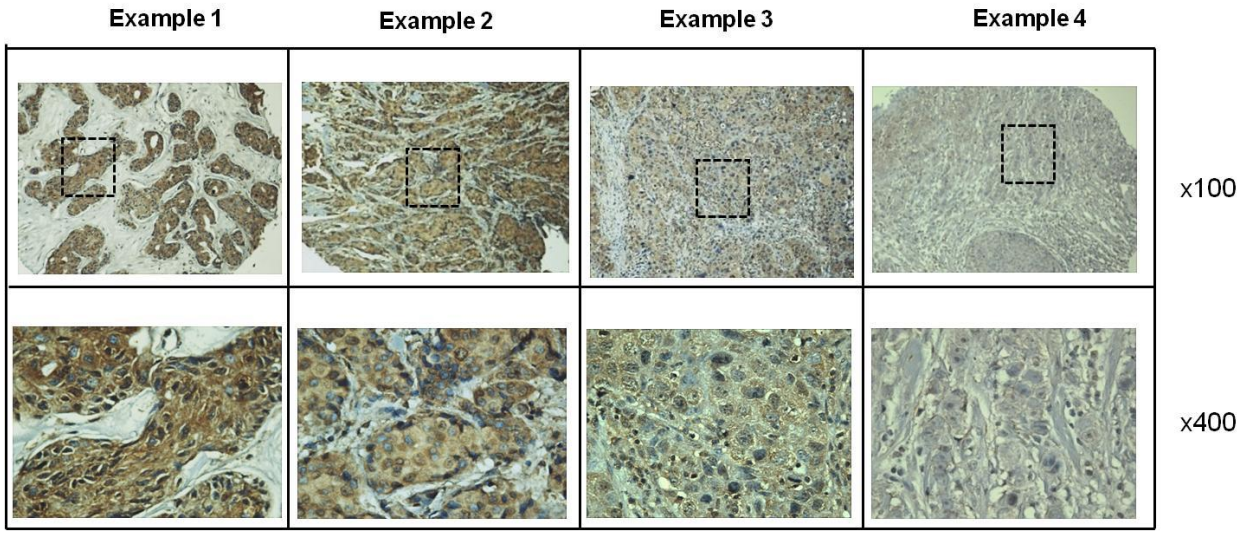


Figure 4: Representative examples of staining of p62/IMP2 in breast tumor tissues. Four representative examples of immunohistochemical staining of p62/IMP2 in breast tumor tissues were shown here: strong positive staining (example 1), moderate positive staining (example 2), weak positive staining (example 3), and negative staining (example 4).

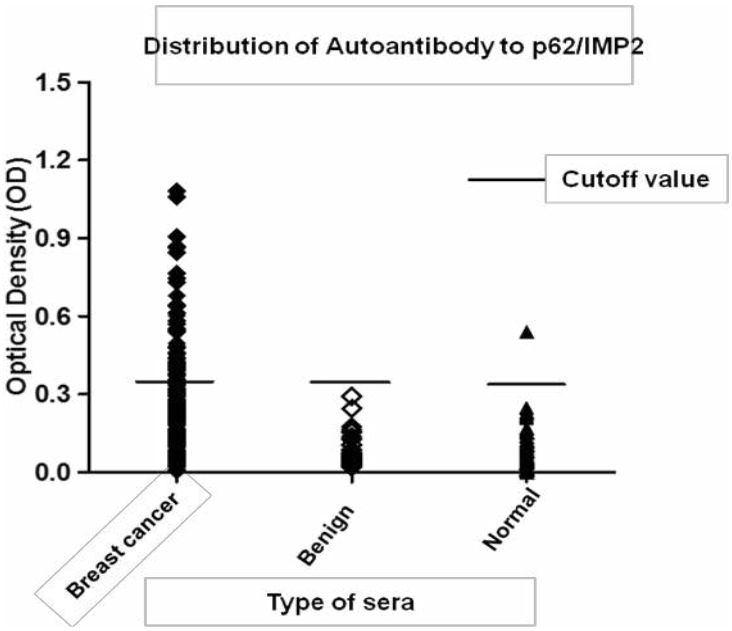


Figure 5: Distribution of autoantibody to p62/IMP2. Titers of autoantibody against p62/IMP2 in sera with breast cancer, sera with benign tumor, and normal human sera. The distribution of antibody titers is indicated as optical density (OD) obtained from ELISA. The mean + 3SD of normal human sera were shown in relationship to all sera samples. Titer of anti-p62/IMP2 in sera of patients with breast cancer was much higher than that observed in sera from patients with benign tumor and sera from normal individuals. Breast cancer: antibody titer to breast cancer patients. Benign: antibody titer to breast benign tumor. Normal: antibody titer to normal human sera.

Chapter 5: The effect of P62/IMP2 on breast cancer progression

5.1 Overview

Based on the discussion in chapter 4, we have known that p62/IMP2 is highly associated with breast cancer from chapter 4. The next question then becomes: what is the cancer biology of p62/IMP2? Is p62/IMP2 just a passer-by induced by other genes or is p62/IMP2 a driver in breast cancer? This functional study can help us to understand the role of p62/IMP2 in breast cancer better. The cancer cell behavior is altered significantly when compared with the behavior of normal cells, such as the manner of cell survival, cell growth, cell migration, and cell adhesion. In chapter 5, we are aiming to explore the effect of p62/IMP2 on breast cancer progression via an in vitro study as well as in vivo study. The results from this chapter will clarify what kinds of cell behavior are affected by p62/IMP2.

5.2 Rationale, experimental design and alternative approach

Specific aim 2: To determine the effect of p62/IMP2 on breast cancer progression

5.2.1 Rationale:

The encoded proteins of target mRNAs of p62/IMP2 contain oncoproteins, receptors, kinases, receptors, and transcriptional factors which have implications in all aspects of cancer progression. p62/IMP2 can keep cancer cell survival and growth by binding to a group of mRNAs, including c-myc, Sp1, and IGF1r. Also, p62/IMP2 has been reported to promote cell migration, cell polarization, and cytoskeleton remodeling by targeting a group of adhesive molecules (CAM1, LIMS2, TRIM54, and LAMB2). Additionally, p62/IMP2 supports cancer stem cells survival and self-renewal by binding to and transporting the transcripts encoded by nuclear DNA to vicinity of mitochondria for translation. In this chapter, we discuss how we tested to see if p62/IMP2 can drive breast cancer progression and investigated what kind of role

p62/IMP2 plays in driving breast cancer with an in vitro study and in vivo study.

5.2.2 Experimental Design & Methods:

We screened a panel of breast cancer cell lines to detect the expression of p62/IMP2 in breast cancer cells, and then identified some p62/IMP2 positive cell lines and p62/IMP2 negative cell lines. Our strategy was to use transfection or siRNA to alternate p62/IMP2 expression in breast cancer cells (knockdown and overexpression) to study the effect of p62/IMP2 on breast cancer.

After getting transfected variants, we performed a series of in-vitro assays: cell proliferation assay, wound healing assay, and cell-matrix adhesion assay. **1) Proliferation assay:** cell proliferation can be monitored via numerous methods. Here we used the sulforhodamine B (SRB) method to detect cell proliferation between the generated variants. Compared with the MTT method, which is also a common way to detect cell proliferation, the results from the SRB method are more accurate, stable and reproducible. **2) Wound healing experiment:** cells were cultured in 6-well plates to confluence as a monolayer. Lines (wounds) in the middle area of wells were scratched and these wounds were photographed at each different time point. Migration areas were measured with the image-plus software to determine which kind of cells migrates faster and make the wound heal faster. **3) cell-matrix adhesion assay:** this assay was used to detect the adhesive ability between cancer cells to the extracellular matrix. Two ECM components (fibronectin and collagen) were coated on 96-well plates respectively. Cells were seeded into coated-wells and incubated for 1 (collagen) or 3 (fibronectin) hours. After washing and staining, the adhesive ability of transfected cells was analyzed by calculating cells attached at the bottom of wells.

5.2.3 Statistical approach:

Three independent experiments were performed. Results were expressed as the mean ± standard error of the mean (SEM). The significance of changes between experimental and control groups were assessed via one-way ANOVA. A $p<0.05$ was considered statistically significant.

5.2.4 Potential problems & alternative approaches:

We expected that we can get some p62/IMP2 positive cell lines and p62/IMP2 negative cell lines for the overexpression and knockdown. We also expected that our data from the in vitro study would indicate that p62/IMP2 promotes breast cancer progression. One potential problem was that p62/IMP2 expression may not be found in the cell lines we screened. In this situation, we believed that the data from tissue microarray and sera of patients should be more reliable because these data comes directly from patients. Cell lines derived from tumors are considered as a good model for lab research. However, there is widespread belief that the growth characteristics as well as the genome background of cells will change in the process of establishing cell lines or after subsequent passage. There are hundreds of breast cancer cell lines established in the world, so we ensured to take more cell lines for the screening. If most of cell lines screened were p62/IMP2 negative, we planned to use overexpression-based transfection for the further functional study. On the other hand, we planned to use knockdown-based transfection for the further functional study. The other potential problem was that different cancer cell lines would have different genetic backgrounds. Therefore working with just one cell line could not demonstrate the real role of p62/IMP2 in breast cancer progression. We planned to work with two or more cell lines by implementing the gain-of-function assay and the loss-of-function assay.

5.3 Results

5.3.1 p62/IMP2 expression in breast cancer cells and Generation of MDA-MB-231 variants, LM2-4 variants, and MCF7 variants with stable overexpression of p62/IMP2

To test the endogenous expression of p62/IMP2 in breast cancer cells, we screened a panel of breast cancer cell lines. Western Blotting results of these 8 different cancer cell lines (SKBr-3, BT474, MCF7, T47D, ZR-75-1, MDA-MB-231, H2N-231, and LM2-4) revealed that the expression of p62/IMP2 was present in the SKBr-3 cell line but not other cell lines (Fig.6.). To investigate the biological role of p62/IMP2 in breast cancer progression, three p62/IMP2 negative cell lines (MDA-MB-231, LM2-4 and MCF7) were transfected with pcDNA3.1-p62 vector and empty vector as control. 1mg/ml G418 was added into the transfected cells to ensure a generation of stable variants expressing of p62/IMP2. The LM2-4 cell line holds higher metastasis potential than MDA-MB-231 cell line, and MCF7 is a cell line with lower metastasis potential compared with MDA-MB-231 cell line. Also, LM2-4 cells and MDA-MB-231 cells share a similar genetic background, which are very different from the genetic background of MCF7 cell line. It is interesting to consider if we can get the same results with the three different cell lines. In this way, the role of p62/IMP2 in regards to breast cancer progression can be tested. More than 30 positive clones were obtained from the three cell lines. Next, two positive clones (one with high expression of p62/IMP2 and the other with moderate expression of p62/IMP2) and one negative clone were selected from the MDA-MB-231, LM2-4 and MCF7 cell lines respectively (Fig.7.). Transfected cells were further confirmed with cell immunoflurosence. The localization of p62/IMP2 was shown in cytoplasm of positive clones (Fig.8.).

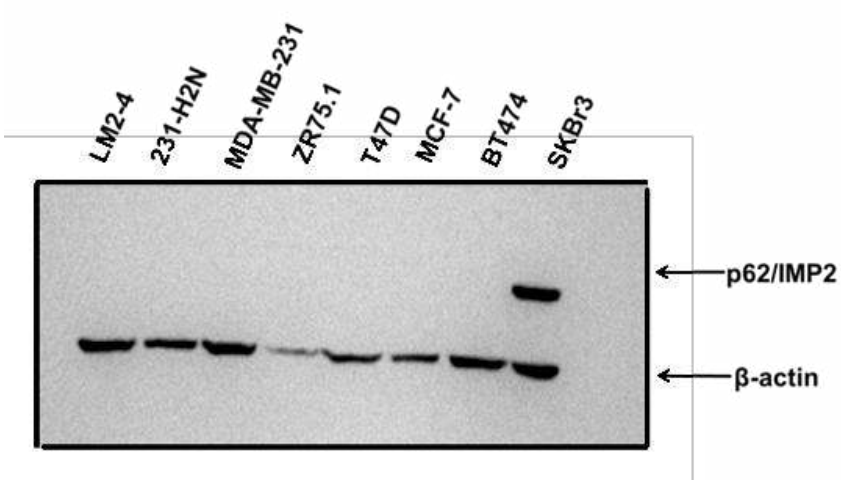


Figure 6: Analysis of p62/IMP2 expression in a panel of human breast cancer cell lines. The panel included 8 different kinds of cell lines. p62/IMP2 was detected to be expressed in the SKBr3 cell line. Cells were harvested and western blotting was performed on the cells lysates using antibodies against p62/IMP2, and beta-actin.

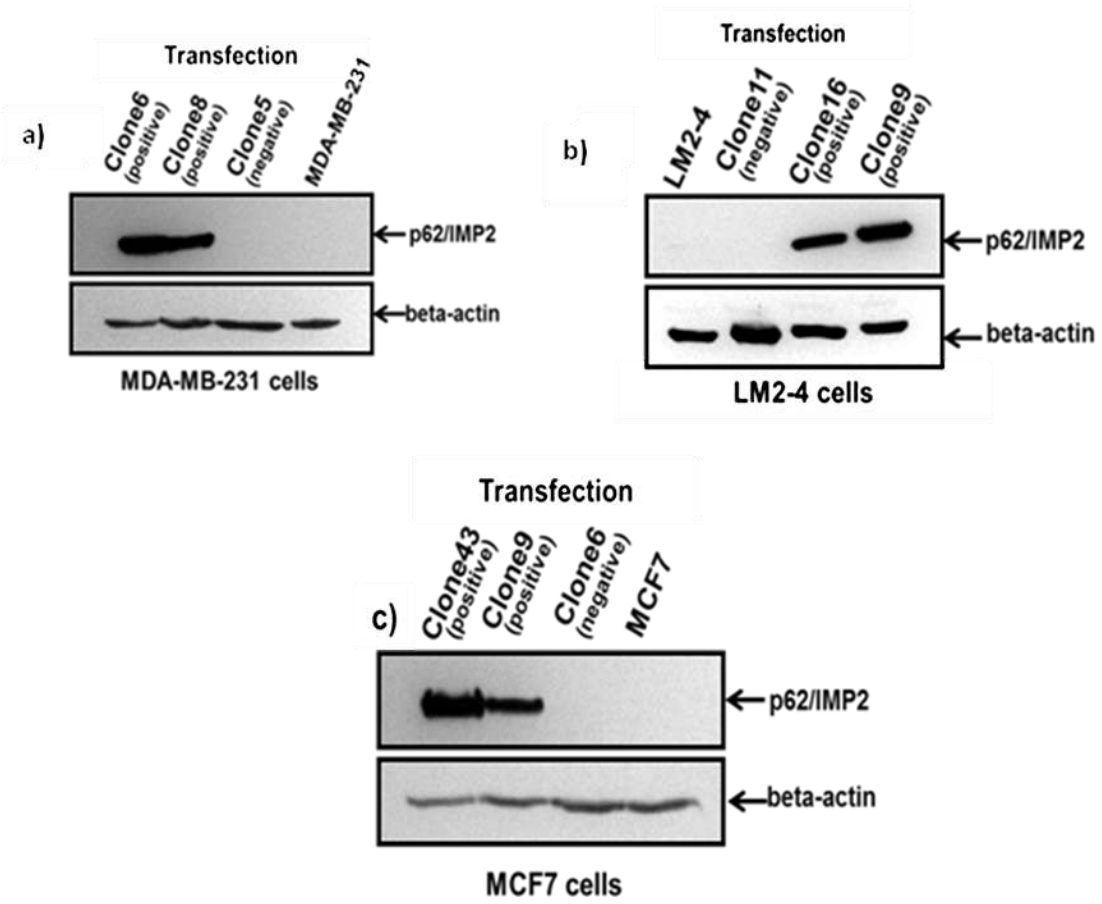


Figure 7: Generated variants with overexpression of p62/IMP2 in breast cancer cell lines. a) Stable transfection of p62/IMP2 into MDA-MB-231 cells. Clone 6 and Clone 8 were p62/IMP2 positive clones. Clone 5 was a p62/IMP2 negative clone. b) Stable transfection of p62/IMP2 into LM2-4 cells. Clone 9 and Clone 16 were p62/IMP2 positive clones. Clone 11 was a p62/IMP2 negative clone. c) Stable transfection of p62/IMP2 into MCF7 cells. Clone 43 and Clone 9 were p62/IMP2 positive clones. Clone 6 was a negative clone. Cells were transfected with IMP2/p62-pcDNA3.1 plasmid. MDA-MB-231 cells or LM2-4 cells or MCF7 cells with empty vector were served as corresponding controls.

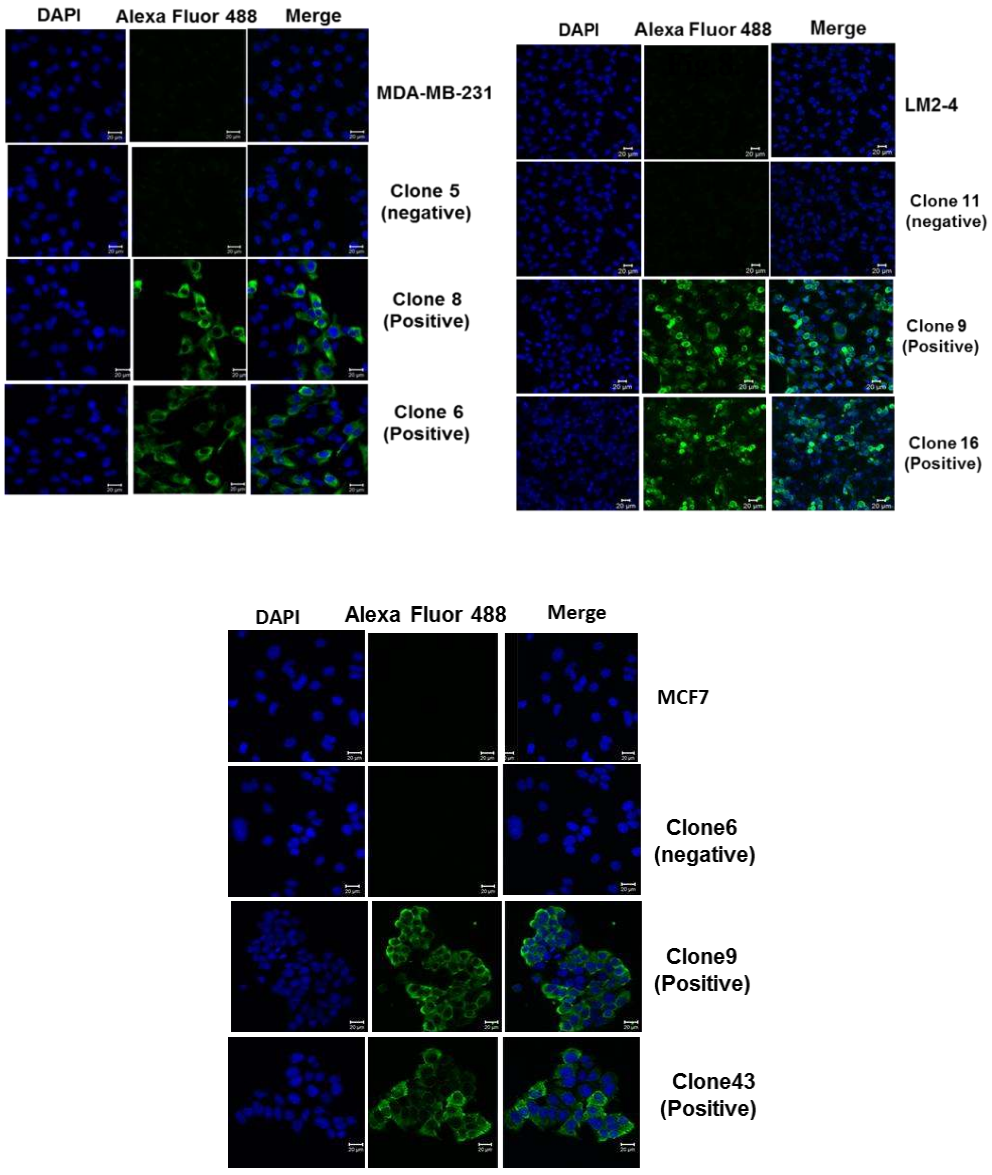


Figure 8: Cell immunofluorescence staining of p62/IMP2 in transfected cells. There is no p62/IMP2 staining in these negative clones and in the three parental cell lines. In these positive clones, the staining of p62/IMP2 is mainly localized to the cytoplasm. Indicated cells were immunostained with a specific anti-p62/IMP2 antibody, and with DAPI.

5.3.2 Overexpression of p62/IMP2 promotes cell migration and reduces cell-extracellular adhesion.

Metastatic tumors are usually correlated with increased cell migration and deregulation of cell adhesion. Thus, the wound healing assay and the adhesion assay were carried out to determine the migratory and adhesive characters between p62/IMP2 positive and negative cells. Our wound healing assay demonstrated that the 'wound' was healed faster by p62/IMP2 positive cells than by negative cells (Fig.9.). Stable overexpression of p62/IMP2 increased wound closure by 50% to 70% (Fig.10.). The result was consistent with other findings, which indicated that p62/IMP2 can induce a strong cell migration.

Three step hypotheses for tumor cells interacted with ECM were proposed: 1) adhesion of cell to the ECM, 2) degradation of the ECM by cellular proteases, and 3) migration/invasion through ECM. Some reports showed that reduced adhesion is necessary for the increase of cell mobility and in favor of cell spread to distant sites. In contrast, other reports showed that increased adhesion to the components of ECM can enhance communication between them and promote cancer cell invasion to the matrigel. Our adhesion study was used to detect the adhesive ability between cells and a defined matrix. Two components of matrix were coated into 96-well plates: collagen and fibronectin. We found that overexpressed p62/IMP2 reduced 30%-50% of cell adhesion to extracellular matrix (Fig.12.). Figure 11 demonstrated that p62/IMP2 positive cells attached to a less extent to the wells, compared to control cells.

Taken together, our data indicated that p62/IMP2 increased cell migration and reduced cell adhesion. In this way, p62/IMP2 may promote breast cancer cells to spread and migrate to distant organs of the body. This suggested that overexpression of p62/IMP2 may contribute to the metastatic behavior of breast cancer cells.

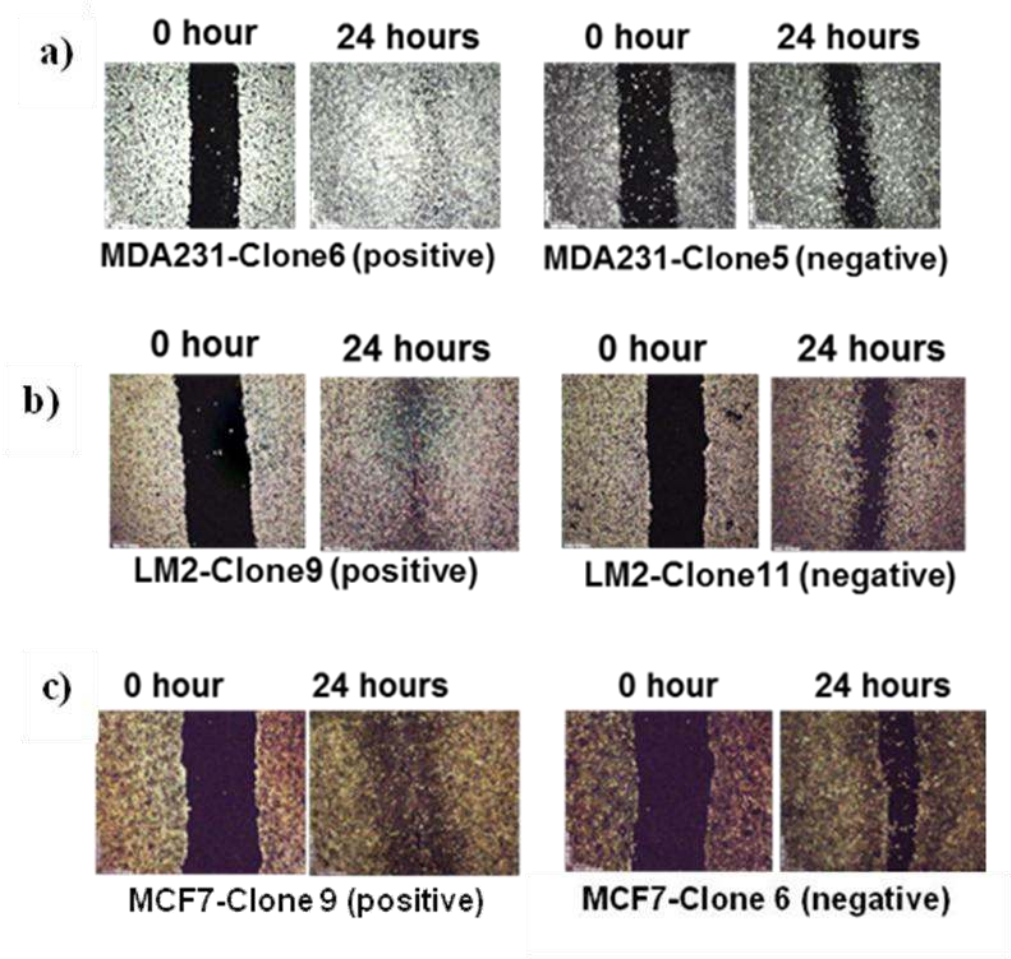


Figure 9: The wound healing images in p62/IMP2 positive and negative cells. Overexpression of p62/IMP2 promoted the migration ability of MDA-MB-231 cells, LM2 cells and MCF7 cells. Wound scratches in different time points allowed for pictures of p62/IMP2 in MDA-MB-231 cells (a), LM2 cells (b) and MCF7 cells (c). After 24 hours, the wound in p62/IMP2 positive clones from three cell lines almost healed. In the negative clones, the wound just become narrower but did not close fully.

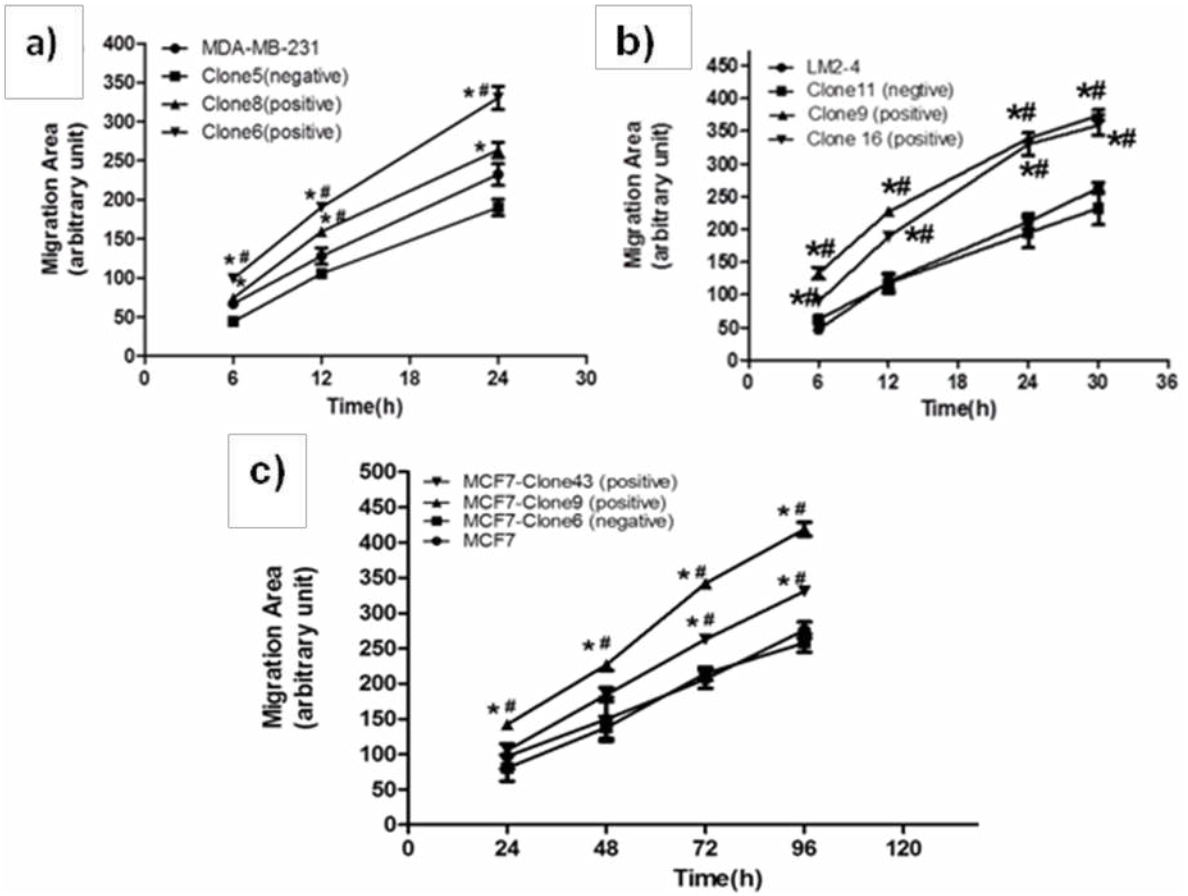


Figure 10: Overexpression of p62/IMP2 promotes migration ability of breast cancer cells. Overexpression of p62/IMP2 promoted the migration ability of MDA-MB-231cells (a), LM2-4 cells (b) and MCF7 cells (c). Wound healing assay showed that there was increased migration ability in p62/IMP2 positive cells compared to negative cells and parental cells. The level of cell migration into the wound scratch was quantified as migration area at each time point. A one-way ANOVA was applied to analyze the results. *, $P < 0.05$ when compared to control clones; #, $P < 0.05$ when compared with parental cells. Values represented mean ± SEM of three independent measurements.

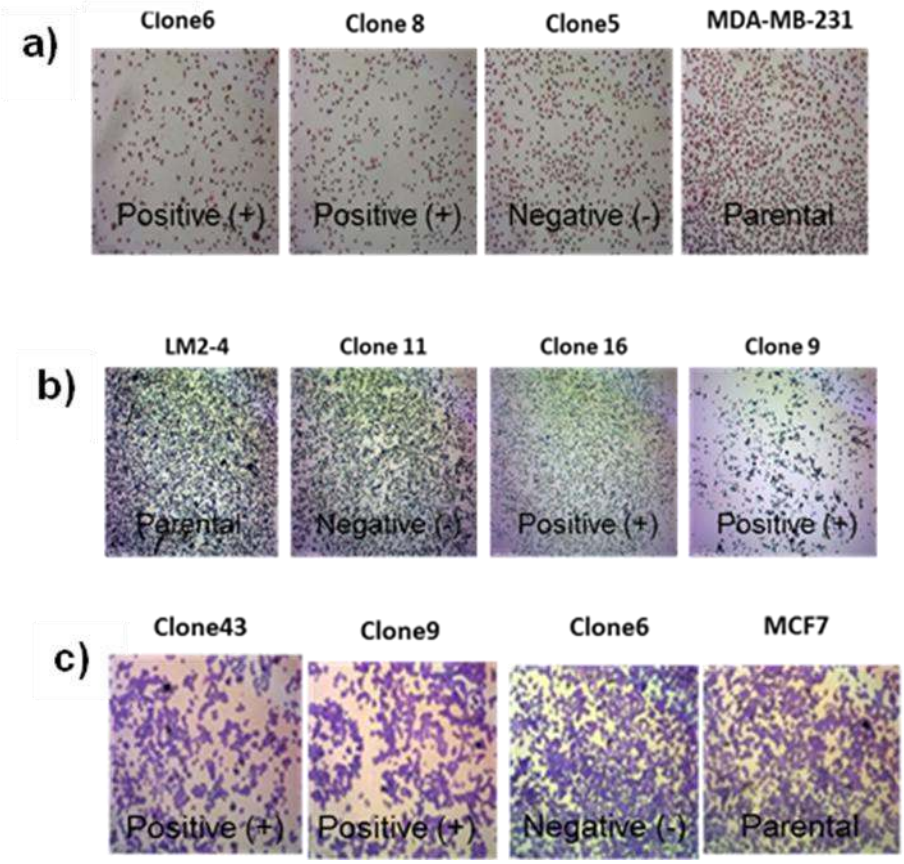


Figure 11: Staining images of attached cells from p62/IMP2 positive and negative clones in the three cell lines. Crystal violet staining images of attached cells from three cell lines: MDA-MB-231cells (a), LM2-4 cells (b) and MCF7 cells (c). From the images, we can directly observe that less p62/IMP2 positive cells were attached at the bottom of wells when compared with the negative cells and parental cells.

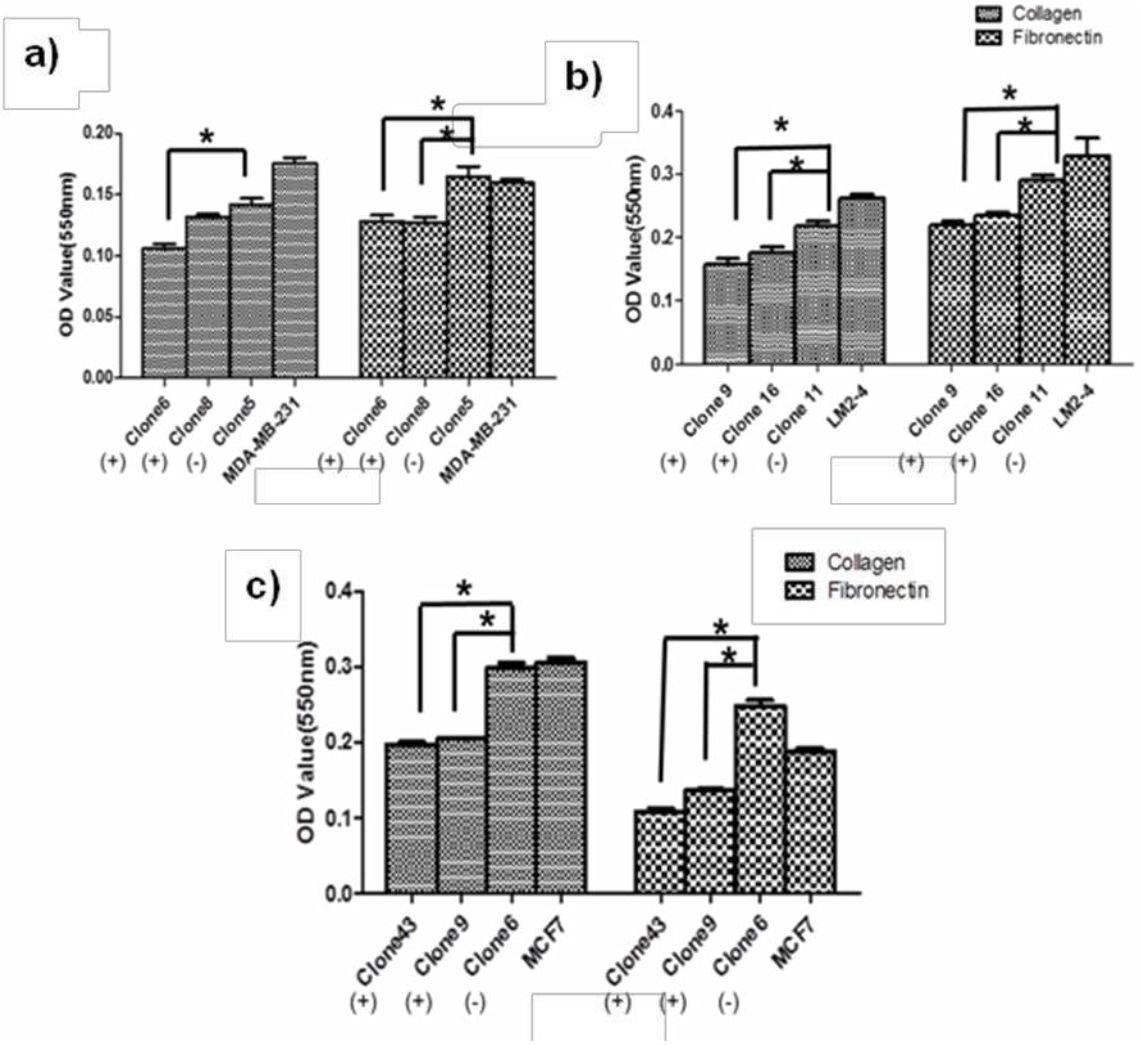


Figure 12: Overexpression of p62/IMP2 reduces adhesion of cells to extracellular matrix. The number of attached cells at the bottom of wells was presented as optical density values following crystal violet staining in the three cell lines: MDA-MB-231 cell line (a), LM2-4 cell line (b), and MCF7 (c). The results showed that overexpression of p62/IMP2 were able to reduce adhesion of cells to extracellular matrix when compared to the negative cells and parental cells. A One-way ANOVA was applied to analyze the results. *, $P < 0.05$ when compared with negative clones. Values represent averages ± SE of three independent measurements.

5.3.3 Overexpression of p62/IMP2 has no effect on cell proliferation

In recent years, it has been reported that p62/IMP2 expression can support cell growth [40], so we measured cell proliferation ability after overexpression of p62/IMP2 in breast cancer cells. However, we did not see any effect of p62/IMP2 expression on breast cancer cell proliferation from the result. The cell growth of variants (p62/IMP2 positive and negative cells) was tracked for 7 days. As shown in Figure 13, the cell growth curve did not suggest any statically significant change after the comparison.

Therefore we concluded that p62/IMP2 may be just specifically involved in cell migration and cell adhesion but not proliferation in the breast cancer progression.

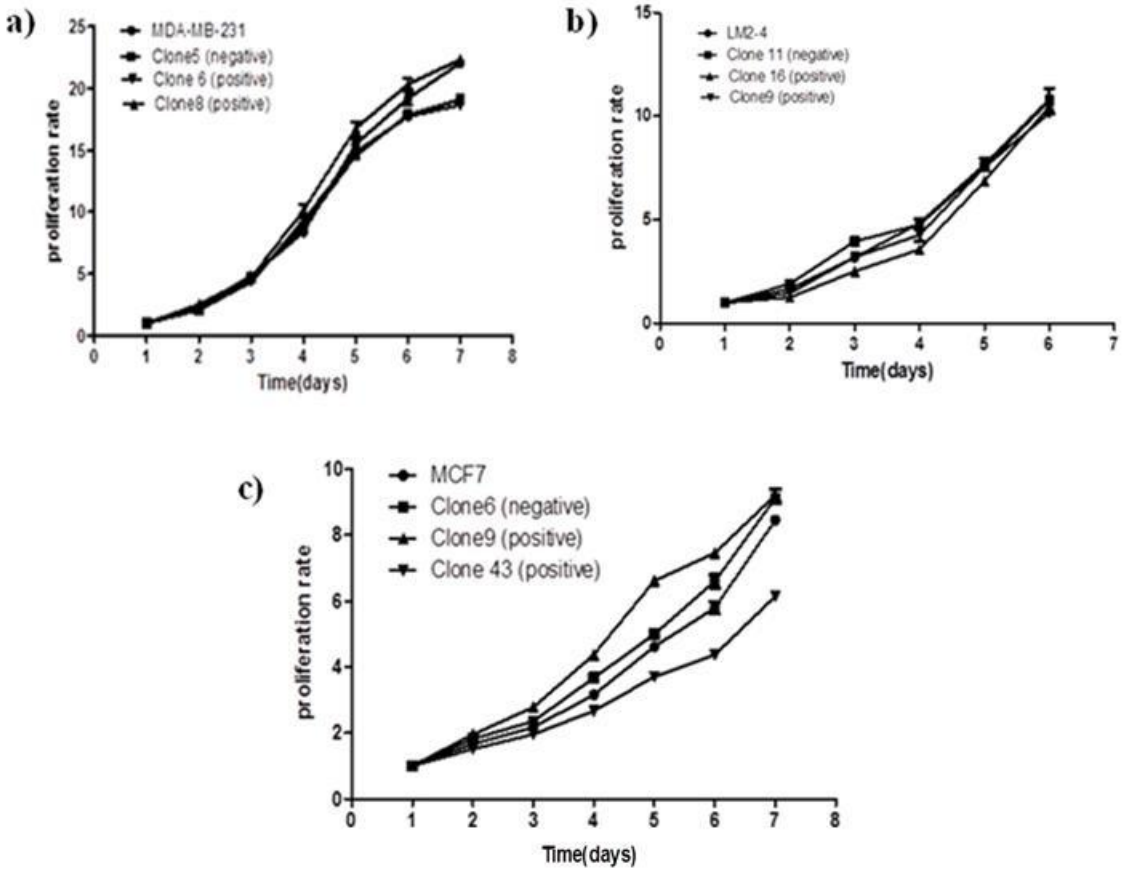


Figure 13: Cell growth curve of p62/IMP2 variants from three breast cancer cell lines. (a) Growth curve of the cell proliferation assay in MDA-MB-231 cells. (b) Growth curve of the cell proliferation assay in LM2-4 cells. (c) Growth curve of the cell proliferation assay in MCF7 cells. There was no obvious effect of p62/IMP2 on the growth of breast cancer cells.

Chapter 6: Identification of targets of p62/IMP2 or related-genes of p62/IMP2 in breast cancer cells

6.1 Overview

Our data from the in vitro study revealed that p62/IMP2 may promote breast cancer metastasis by increasing cell migration and reducing cell adhesion. As to cell-matrix adhesion, some researchers believe that the increase of adhesion can support the talking between cancer cells and the extracellular environment, which contributes to activation and transmission of metastasis signaling in cancer cells. Other reports suggested that the reduction of adhesion promotes cells to spread to other parts of the body. Our conclusion that p62/IMP2 reduces cell-matrix adhesion has interested us on the mechanisms behind it. In this chapter, we explore how p62/IMP2 regulates breast cancer metastasis by controlling the adhesion of breast cancer cells. Additionally we also attempt to identify some targets of p62/IMP2 specific to breast cancer metastasis.

6.2 Rationale, experimental design and alternative approach

Specific aim 3: Identification of targets of p62/IMP2 related with breast cancer metastasis.

6.2.1 Rationale:

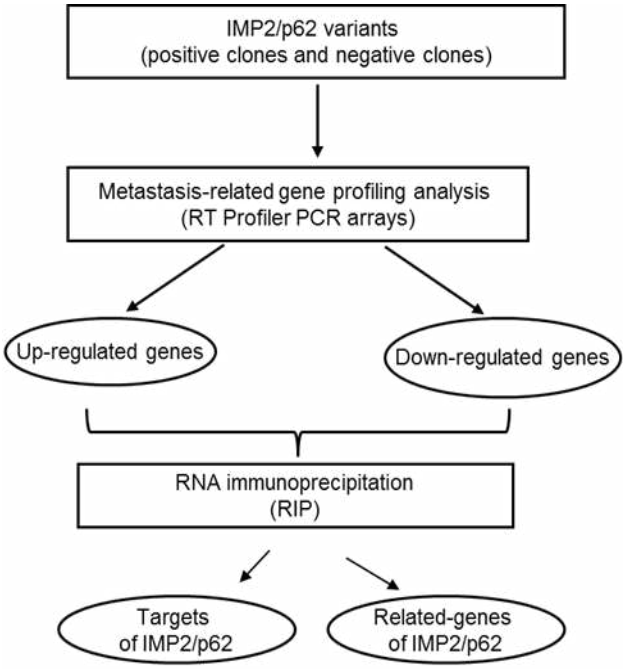
Members in IMP family can bind to more than one hundred mRNAs in different cellular contexts, and which mRNAs can be captured depends on the different cellular context. For example, the homolog IMP1 binds to IGF2 mRNA in rhabdomyosarcoma cells, but binds to CD44 in Hela cells. Target mRNAs binding to p62/IMP2 is enhanced in glioblastoma cancer stem cells with hypoxic conditions. In addition, many targets of IMP are mRNAs of signaling proteins. IMP1 can bind with MAPK4 mRNA and PTEN mRNA in U2OS cells to regulate their phospholipid-dependent signaling networks. Here, we will attempt to identify new

targets of p62/MP2 or p62/IMP2 related genes, and we will try to investigate the specific function of p62 in signaling pathways. Our approach of identifying novel targets is to mainly apply for metastasis-related gene profiling analysis because in this way, we can easily and reliably analyze expression of a focused panel of genes.

6.2.2 Experimental Design & Methods:

Our approach in identifying novel targets was to use metastasis-related gene profiling analysis (see flow Chart in the right panel: Fig 15). RNA was extracted from p62/IMP2 positive clones and negative clones, and then total RNA was reversely transcribed into cDNA. The "Human Extracellular Matrix & Adhesion Molecules PCR Array" was used to quantify changes in gene expression. The Human Extracellular Matrix & Adhesion Molecules PCR Array contains ECM proteins including basement membrane constituents, collagens, and other genes defining ECM structure. Matrix and other metalloproteinase that remodel the ECM as well as their inhibitors were also included. This Matrix & Adhesion array also represented integrins, selectins, cell-adhesion molecule family members (ICAM, ECAM, NCAM, PECAM, and VCAM), and other genes which are important to cell adhesion and cytoskeleton bridging (such as the catenins). qPCR was performed on the MyiQ real-time PCR detection system (Bio-Rad) with SYBR Green qPCR mastermix (SA Biosciences, Qiagen) according to the manufacturer's protocol.

Figure 14: The flow chart of identifying the novel candidates of p62/IMP2



Candidate genes from PCR arrays were selected and validated by RNA immunoprecipitation (RIP) to see if they were targets of IMP2/p62 or just related genes of IMP2/p62. RNA immunoprecipitation was briefly performed using a RNA immunoprecipitation Kit (MBL international). Cells were harvested by adding RIP lysis buffer. Clear supernatant containing IMP2/p62, IgA beads, and IMP2/p62 antibody, were mixed to perform immunoprecipitation. After washing and separation, RNAs binding to IMP2/p62 were eluted and quantified as well as transcripted. PCR with the primers for candidate genes were performed to examine whether their mRNAs were coimmunoprecipitated.

6.2.3 Statistical approach

Gene profiling analyses was performed a minimum of three times in independent experiments. The deta deta t was used for data analysis. Fold changes are calculated for each gene as the difference in gene expression between cells by using the RT Profiler qPCR-array data analysis software.

6.2.4 Expected outcome and Potential Pitfalls

We expected that we could identify some novel target transcripts of p62/IMP2 in breast cancer cells. Every RT² Profiler PCR Array profiles the expression of 84 key genes involved in cell motility or cell adhesion to matrix. However, it does not contain all the related genes. A complementary method to other PCR Assays should be added, such as The Human Focal Adhesions RT² Profiler PCR Array.

6.3 Results

6.3.1 p62/IMP2 can bind to c-myc mRNA in the MDA-MB-231 cells and contribute the increase of c-myc synthesis.

It is well known that c-myc mRNA is an important target of p62/IMP2. To verify if p62/IMP2 is able to bind c-myc in breast cancer cells, we pulled down the p62/IMP2-mRNAs complex via RNA immunoprecipitation. By the RIP and PCR, we found that p62/IMP2 can bind to c-myc mRNA in our generated MDA-MB-231 cells (Fig.15.). Also both c-myc mRNA abundance (Fig. 16a and Fig.16b) and c-myc protein amounts (Fig.16c) were found to increase with overexpression of p62/IMP2 in breast cancer cells, which indicated that this binding can contribute to the stabilization of c-myc mRNA and the increase of c-myc protein. This data verified that p62/IMP2 can bind to its targets and does work in breast cancer cells.

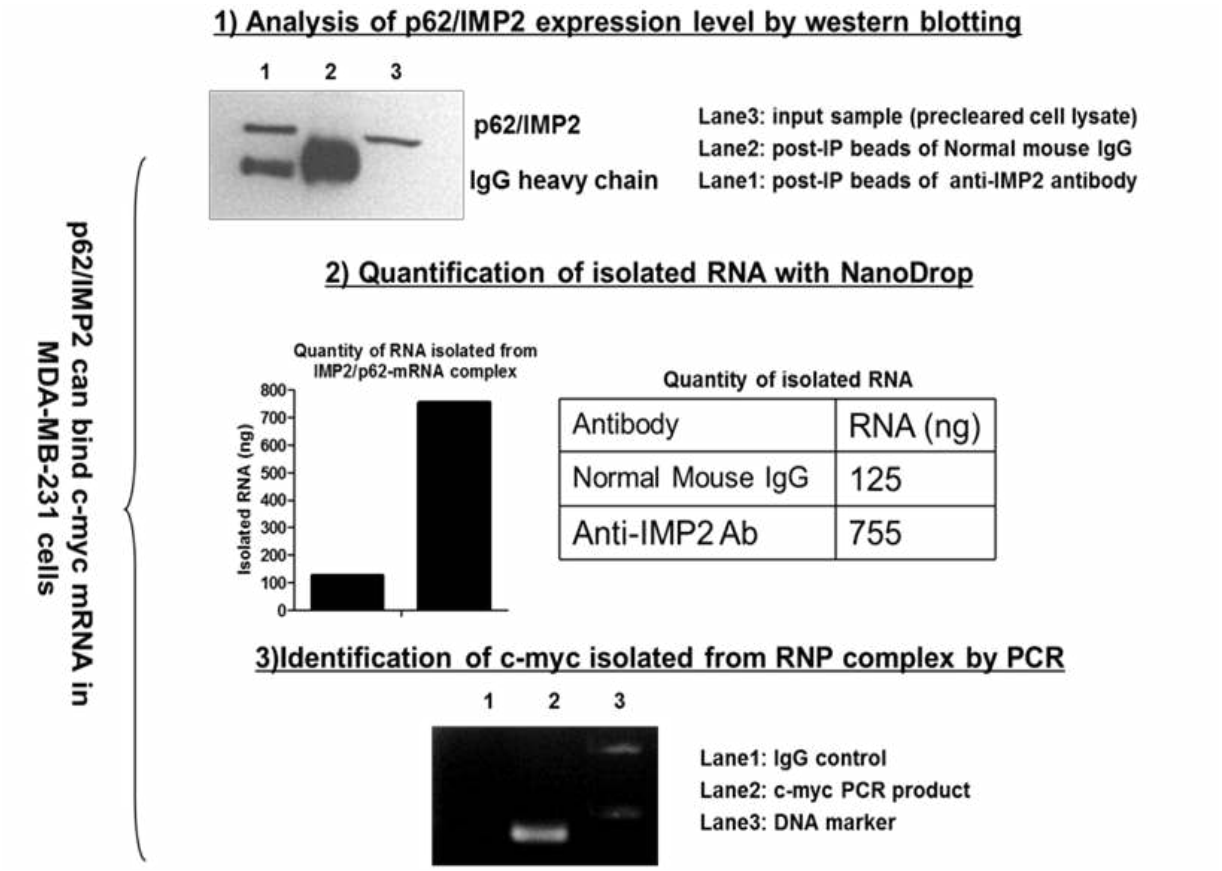


Figure 15: p62/IMP2 can bind c-myc mRNA in MDA-MB-231 cells. After pulling down the whole p62/IMP2-targets complex (1) and separating target mRNAs from p62/IMP2 protein and purifying target mRNAs, we got 755ng mRNAs (2) for reverse transcription as a cDNA pool. Then we used a primer of c-myc to perform the normal PCR and got the band on the gel, which indicated that c-myc mRNA is a target of p62/IMP2 (3).

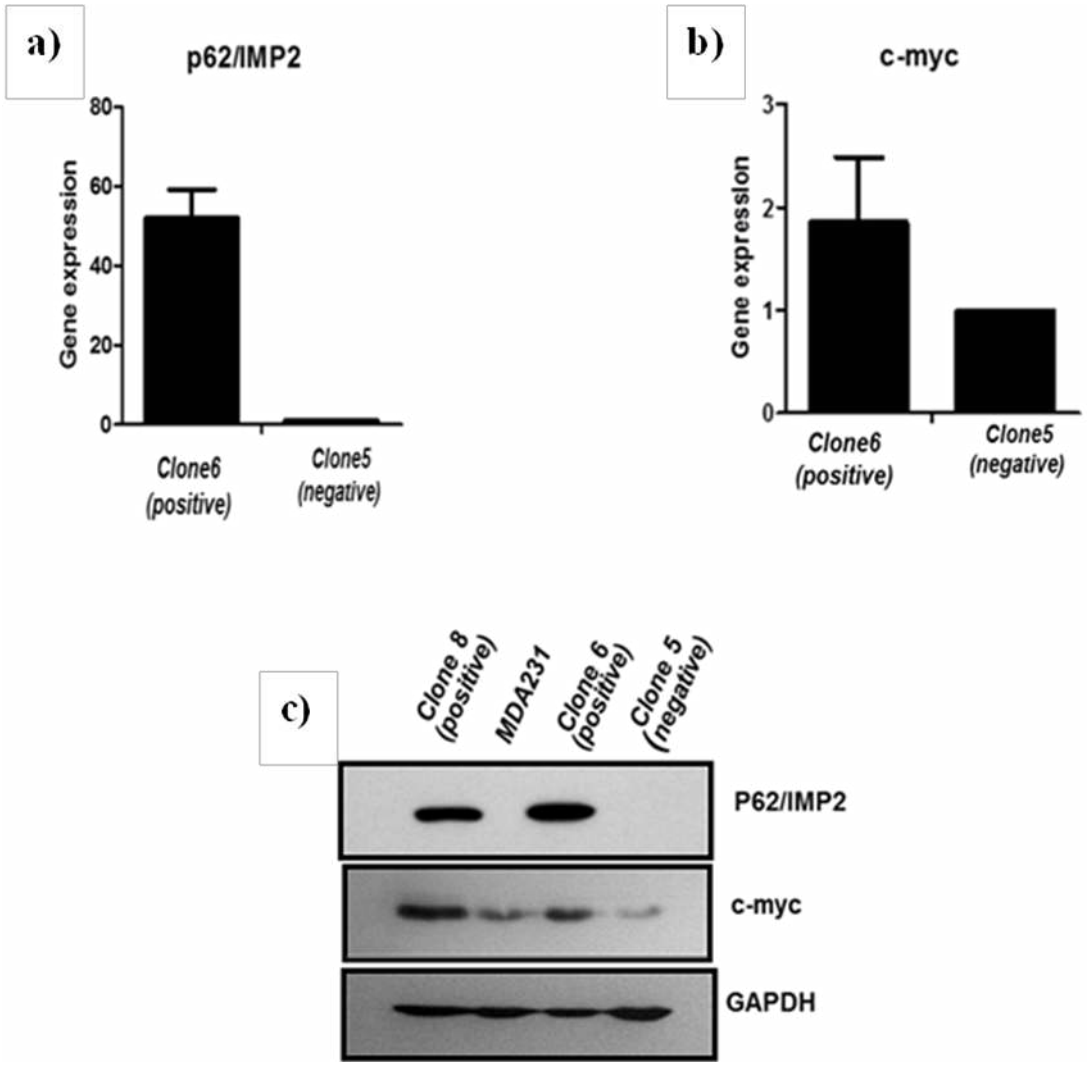


Figure 16: Up-regulation of c-myc by overexpression of p62/IMP2 in MDA-MB-231 cells. mRNA level of c-myc was analyzed by real-time PCR in MDA-MB-231 cells, and results showed that c-myc mRNA was increased with the overexpression of p62/IMP2 (a & b). Protein level of c-myc was analyzed by Western Blotting in MDA-MB-231 cells. The c-myc protein was also increased with the overexpression of p62/IMP2 (c). So p62/IMP2 can does work in MDA-MB-231 cells, and p62/IMP2 can increase c-myc expression in the RNA level as well as in protein level.

6.3.2 Changes in human extracellular matrix and adhesion molecular expression in response to overexpression of p62/IMP2

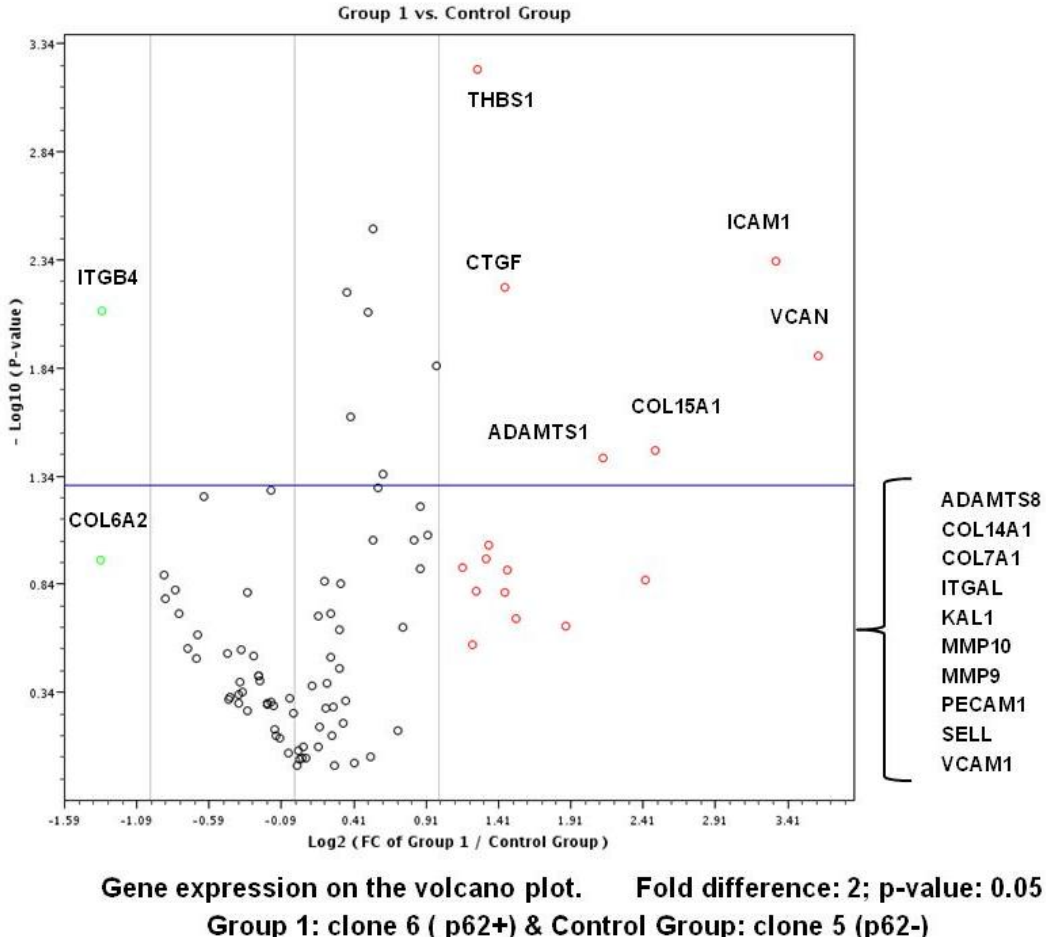
The local microenvironment of cancer cells plays an important role in cancer progression, and its major component is the extracellular matrix (ECM). The deregulation of ECM is very common in cancer, especially in cancer metastasis. Abnormal ECM can promote cancer metastasis by affecting cell migration and cell adhesion directly. Since our study in specific aim 2 suggested that p62/IMP2 can reduce cell adhesion to promote cancer metastasis, we have an interest on uncovering the underlying mechanisms, particularly in regards to human breast cancer. The Human Extracellular Matrix & Adhesion Molecules RT² Profiler PCR Array was decided to perform with p62/IMP2 positive/negative cells to see which genes work together with p62/IMP2. This array profiles the expression of 84 genes critical for cell-cell and cell-matrix interactions, including basement membrane constituents, collagens, and genes defining ECM structure, metalloproteinases and their inhibitors.

Out of 84 genes, 16 were found to be upregulated with a difference greater than 2 fold with overexpression of p62/IMP2. Only 2 were found to be down-regulated (Fig.17.). Among them, the p-value of 7 genes, including 6 up-regulated genes and 1 down-regulated gene, was less than 0.05 (Table 6). THBS1 was one of the candidates in the array, and its mRNA was increased 2.4 fold. It is well-known that the turnover of THBS1 mRNA can be increased by c-myc expression. This information validated the result of our qPCR array as reliable. In our additional qPCR experiment, the increased mRNAs of verscian, CTGF, ADAMTS1, THBS1 and COL7A1 were confirmed again with our primers in MDA-MB-231 cells (Fig.19a).

In the LM2-4 cells, we also found the increased expression of c-myc with the overexpression of p62/IMP2 (Fig.18). Therefore we tried to validate the results of qPCR array

from MDA-MB-231 cells in LM2-4 cells, and CTGF, ADAMTS1, VCAN, and COL7A1 were confirmed to be increased in mRNA level with overexpression of p62/IMP2 (Fig.19b).

Human Extracellular Matrix and Adhesion Molecules PCR array in MDA-MB-231 cells



Gene expression on the volcano plot. Fold difference: 2; p-value: 0.05
Group 1: clone 6 (p62+) & Control Group: clone 5 (p62-)

Figure 17: Volcano plots analysis of adhesion qPCR array. This is a volcano plot that showed the significant gene expression changes with overexpression of p62/IMP2. Down-regulated genes are described as green in color, and up-regulated genes are in red. Group 1: clone 6 (p62+) & Control Group: clone 5 (p62-).

Gene	Description	Fold Regulation	P value	Function
VCAN	Versican	12.3084	0.013	an anti-adhesive molecule. Increased versican expression is often observed in tumor growth and metastasis in tissues
CTGF	Connective tissue growth factor	2.735	0.006	cell adhesion and migration, skeletal development, tissue wound repair, fibrotic disease, Diabetic nephropathy
ADAMTS1	ADAM metallopeptidase with thrombospondin type 1 motif, 1	4.3819	0.038	necessary for normal growth and fertility. associated with inflammatory processes and development of cancer cachexia
THBS1	Thrombospondin 1	2.3976	0.0006	Myc increases turnover of its mRNA. It plays roles in platelet aggregation, angiogenesis, and tumorigenesis.
COL15A1	Collagen, type XV, alpha 1	5.6499	0.034	To adhere basement membranes to underlying connective tissue stroma. Associated with muscle and microvessel deterioration
ICAM1	Intercellular adhesion molecule 1	10.067	0.005	Stabilization of cell-cell interactions and facilitating leukocyte endothelial transmigration.
ITGB4	Integrin, beta 4	-2.5171	0.0078	To mediate cell-matrix or cell-cell adhesion, and transduced signals that regulate gene expression and cell growth

Other Genes	Fold Regulation	P value
ADAMTS8	2.735	0.156
COL14A1	2.392	0.154
COL7A1	2.5052	0.11
ITGAL	3.6762	0.225
KAL1	2.5401	0.094

Other Genes	Fold Regulation	P value
MMP10	5.3575	0.136
MMP9	2.8843	0.207
PECAM1	2.3428	0.272
SELL	2.2422	0.120
VCAM1	2.7796	0.124

Other Genes	Fold Regulation	P value
COL6A2	-2.5346	0.110

Table 6: The candidate genes selected from adhesion qPCR array in MDA-MB-231 cells. The expression of 18 genes was shown to be changed with overexpression of p62/IMP2. 7 genes from them were selected as candidates for the follow-up study (fold difference: 2; p-value: 0.05).

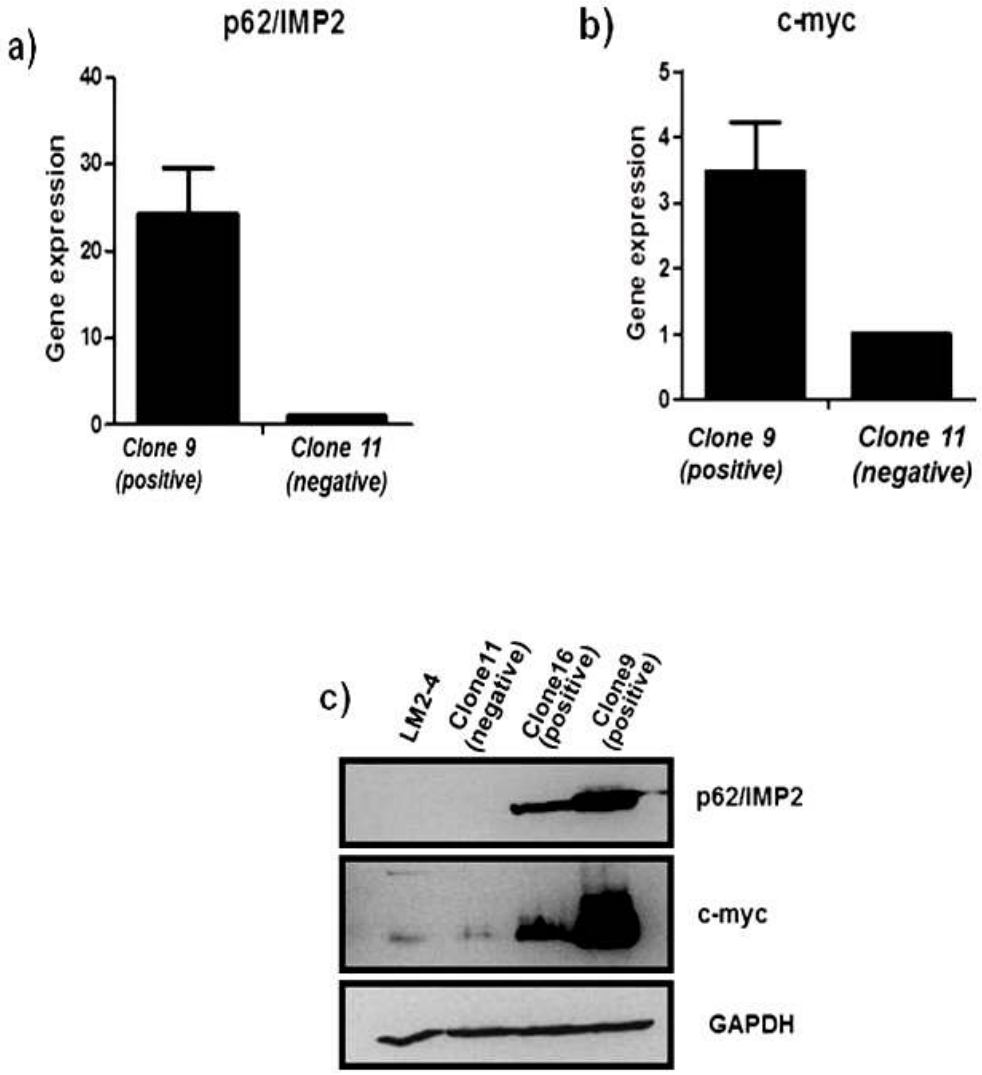


Figure 18: Up-regulation of c-myc by overexpression of p62/IMP2 in LM2-4 cells. mRNA level of c-myc was analyzed by real-time PCR in LM2-4 cells. The c-myc mRNA was increased with the overexpression of p62/IMP2 (a & b). Protein level of c-myc was analyzed via Western Blotting in LM2-4 cells. The c-myc protein was also increased with the overexpression of p62/IMP2 (c).

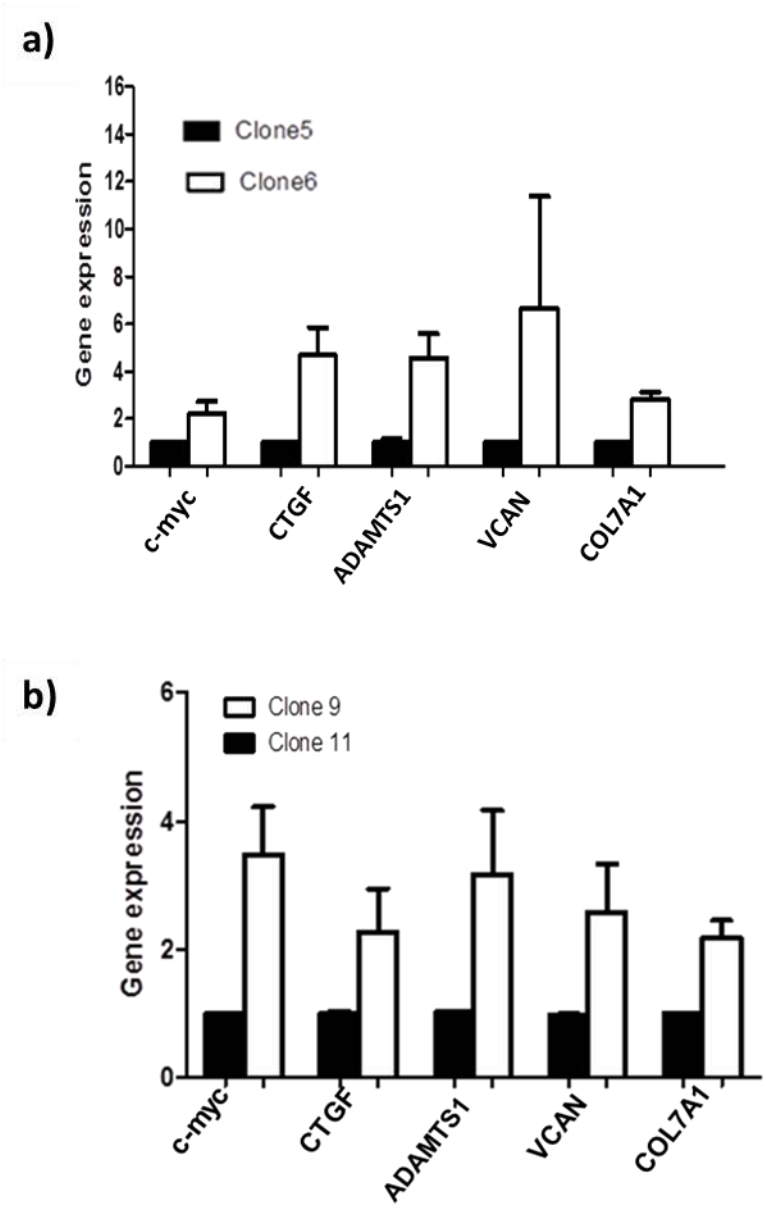


Figure 19: The evaluation of candidates from MDA-MB-231 cells and LM2-4 cells. Increased mRNA of VCAN, CTGF, ADAMTS1, and COL7A1 were confirmed again with our primers in MDA-MB-231 cells (a). Also in the LM2-4 cells, we validated the results from MDA-MB-231 cells in LM2-4 cells, and CTGF, ADAMTS1, VCAN, and COL7A1 were confirmed to be increased in mRNA level with overexpression of p62/IMP2 (b).

6.3.3 p62/IMP2 binds CTGF mRNA and stabilizes the transcript

CTGF is well-known to contribute to the process of tissue wound repair. Some functional features of it are very similar to that of p62/IMP2. CTGF plays important roles in many biological processes, including cell migration and adhesion, tissue development and stem cell pluripotency. In addition, it plays significant roles in many human biological disorders like diabetes, and cancer. It has been known that CTGF expression can be regulated by post-transcriptional regulatory molecules, including microRNAs (miR-18a) in 3'-UTR, which will result in the repressed translation of CTGF or the degradation of its transcripts. Interestingly, in previous studies, p62/IMP2 as an mRNA-binding protein can bind its target mRNAs within 5' or 3' untranslated regions (UTRs). This information raised the possibility that p62/IMP2 could bind CTGF mRNAs directly and competitively. Therefore, we mapped that CTGF was a novel target of p62/IMP2 in breast cancer cells using an RNA immunoprecipitation assay and the experiment of mRNA half-life detection. The results illustrated in Figure 20a show that CTGF can be pulled down with p62/IMP2, but not GAPDH. The results in Figure 20c demonstrated that this binding stabilized the mRNA of CTGF. The well-known target of p62/IMP2, c-myc mRNA is viewed as a positive control here (Fig. 20a and 20b). Our data indicated that CTGF mRNA is a novel target of p62/IMP2 in breast cancer cells and that this binding can stabilize mRNA of CTGF.

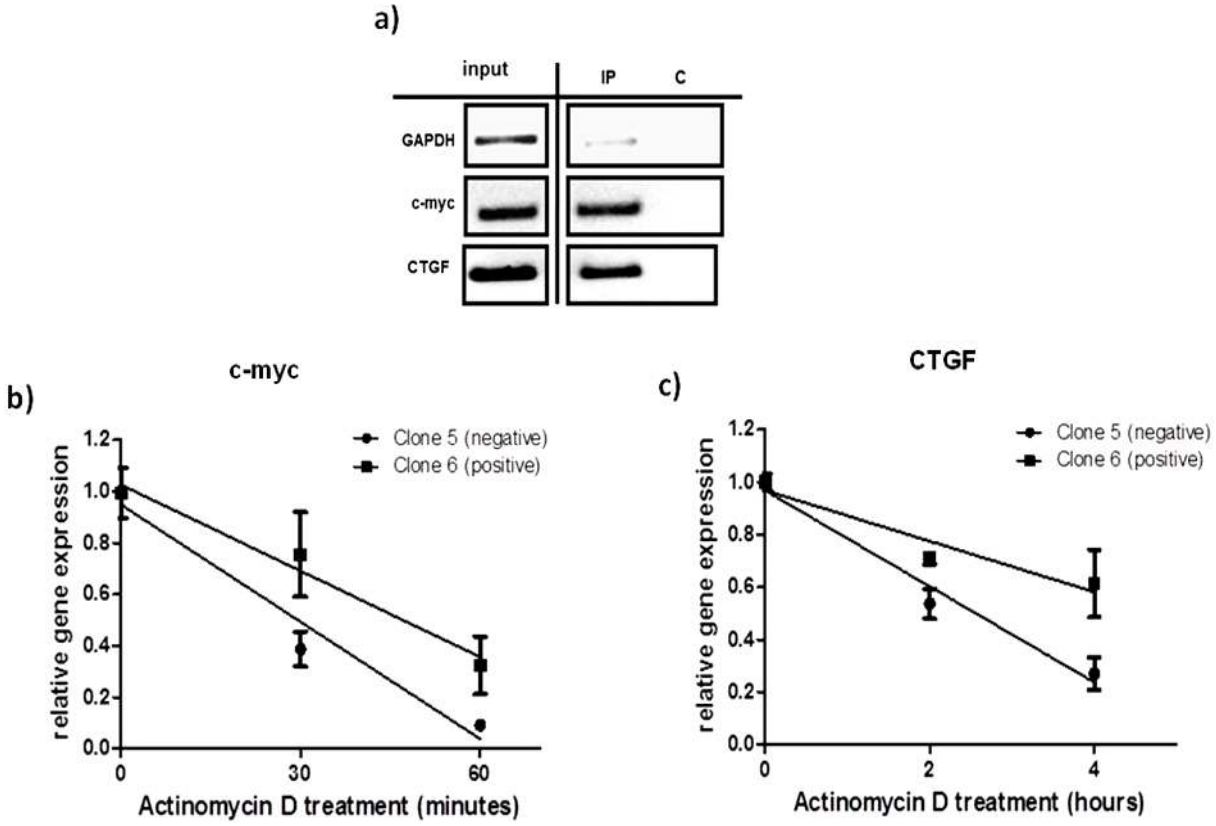


Figure 20: CTGF mRNA is a novel target of p62/IMP2. a) In the MDA-MB-231 cell line, p62/IMP2 bound to mRNA of CTGF and c-myc but not GAPDH. b) p62/IMP2 regulated the stability of c-myc mRNA in MDA-MB-231 cells. In the negative clone 5, the half-life of c-myc mRNA is about 30 minutes, but in the positive clone 6, it is about 50 minutes. c) p62/IMP2 regulated stability of CTGF mRNA. The half-life of CTGF mRNA is about 2 hours in negative clone 5, but it is about 4 hours in the positive clone 6.

Chapter 7: Discussion and Future Direction

7.1 Discussion

One of our main finding in the present study is that p62/IMP2 expression is highly associated with breast cancer development. Our data illustrates the expression of p62/IMP2 in breast cancer tissues using the IHC microarray analysis, and the detection of anti-p62/IMP2 autoantibody in sera from patients with breast cancer as measured by ELISA. Our IHC analysis found high levels of p62/IMP2 expression in breast cancer tissues when compared to adjacent normal tissues. The ELISA data showed a high frequency of anti-p62/IMP2 autoantibody in sera from patients with breast cancer compared to it in the sera from healthy individuals and patients with breast benign tumor. The overexpression of p62/IMP2 and the detection of p62/IMP2 autoantibodies have been reported to occur in several types of cancers. p62/IMP2 is developmentally regulated and is expressed in fetal tissues but not in adult livers, and aberrantly expressed in some hepatocellular carcinomas. Detectable autoantibody to p62/IMP2 was found to be present in 21.1% of HCC patients from China but not in patients with precursor conditions such as chronic hepatitis and liver cirrhosis. In 2013, Liu et al. studied the expression of p62/IMP2 in colon cancer tissues by IHC. Of 64 colon cancer tissue specimens examined, 48 tissues (75.0%, 48/64) expressed p62/IMP2, while normal colon tissues did not show any expression (0% 0/34) [64]. In a study of 82 patients with digestive canal tumors , 38.6% of patients had sera that tested positive for autoantibodies to p62/IMP2, and 33.7% of them had metastatic disease, which suggests that high levels of autoantibody to p62/IMP2 might correlate with metastasis [62]. Detectable antibody to p62/IMP2 has also been found in other types of

cancers, such as ovarian cancer and esophageal cancer [61]. Our results reveal that p62/IMP2 is overexpressed in breast cancer (69.0%) and that the frequency of detectable autoantibody to p62/IMP2 (29.0%) in the sera of breast cancer patients was significantly higher than the frequency in sera from normal individuals (1.0%). Several studies have demonstrated that autoantibody in sera from patients with cancers is produced by the immune system which is stimulated by some cellular proteins. These cellular proteins were generally called tumor-associated antigens (TAAs), of which may originate via mutated proteins or abnormally expressed proteins in cancer. The anti-TAA autoantibody and its potential applications in cancer have been extensively investigated. Autoantibody is stable and can be detected by using ELISA which is noninvasive, easily acceptable way in clinical. More importantly, the significant elevation of autoantibody levels also correlates with the occurrence of some cancers so the detection of the autoantibody can be used for diagnosing early stages of some cancer types. In future studies, serial serum samples (the collection of sera from an individual every year for several years before and after the diagnosis of breast cancer) should be examined to see if an autoantibody to p62/IMP2 can be viewed as a real biomarker in the early diagnosis of breast cancer.

The functional studies of p62/IMP2 showed that this protein can promote cancer progression [67, 66, 40]. However, there is not much research that discusses the effect of p62/IMP2 on breast cancer progression, so it would be interesting to test whether p62/IMP2 expression promotes progression of breast cancer in vitro. To explore the role of p62/IMP2 in breast cancer in vitro, the growth ability, the migration ability, and the ability to adhere to the extracellular matrix of cells with overexpressing p62/IMP2 were examined in this study. Our data demonstrated that p62/IMP2 can increase cell migration and reduce cell adhesion to the

extracellular matrix, but it has no obvious impact on cell proliferation. Increased cell migration is important consequence of EMT [74, 75]. To study cell migration we used a scratch wound-healing assay. It is generally believed that EMT is an important event in cancer metastasis. EMT is also a physiological process that occurs during embryonic development and wound healing[76]. During wound healing, cells undergo a transient EMT in order to migrate into the wound site and close the gaps in the epithelial cell sheets that were created by the wounding process. Also our result of adhesion assay suggested that the reduced adhesion of breast cells with overexpressed p62/IMP2 to extracellular matrix may promote breast cancer cells to leave the primary site and spread to distant organs of the body.

In a recent study of breast cancer, Gui et al found that adhesion of primary breast cancer cells from node-positive women to each of fibronectin and collagen was significantly less than that in node-negative patients[77]. Many proteins play important roles in this process. Some proteins such as SOX2 (a regulator of embryonic stem cells) and CO-029 (also a tumor-associated antigen), can increase the migratory and metastatic potentials and decrease adhesion ability of serous ovarian carcinoma cells [78, 79]. Many proteins like them can contribute to the disorganization of ECM responsible for the changed adhesion between cells and ECM. In fact, the process of EMT and disorganization of ECM are independent of each other sometimes, while in most cases the deregulated ECM can promote EMT and verse versa. Other reports stated that ECM can modulate EMT to promote cancer metastasis [80]. ECM surrounding tumor cells is responsible for activating and adjusting intracellular signaling to drive EMT [81]. In short, our data from in vitro study indicated that p62/IMP2 may be an important regulator in breast cancer metastasis by increasing the migration ability of breast cancer cells and reducing the adhesion ability of breast cancer cells to extracellular matrix.

To further explore how p62/IMP2 regulates wound healing and adhesion, a Human Extracellular Matrix & Adhesion Molecules RT² Profiler PCR Array was carried out to observe mRNA changes regulated by p62/IMP2. We found that most of changed genes are upregulated (such as CTGF, VCAN, ADAMTS1, and COL7A1) by overexpression of p62/IMP2. How p62/IMP2 regulates these genes has yet not to be reported. Since p62/IMP2 is an mRNA binding protein, the main function of p62/IMP2 is to stabilize its target mRNAs and upregulate their expression. Therefore, one possibility is that the mRNAs of upregulated genes with overexpression of p62/IMP2 in breast cancer cells are bound to p62/IMP2 proteins. Among them, CTGF was chosen to test such a possibility because the half-life of its mRNA is short. In our experiment, CTGF mRNA is indicated to be a target of p62/IMP2 by the mRNA half-life assay and RIP assay. Because of the role of CTGF on cell migration process, studies about the expression of CTGF correlated to cancer metastasis have increased since 2013[82]. It has been shown that overexpression of CTGF was related with potential metastasis in pancreatic cancer, lung cancer, gastric cancer, and osteolytic metastasis of breast cancer [83-85]. In 2014, CTGF was firstly demonstrated to be unregulated in p62/IMP2 transgenic mice and the unregulated expression of CTGF is TGF-beta-independent[86]. They suggested that IL13 might be involved in the up-regulation. However, our data showed that p62/IMP2 can bind to mRNAs of CTGF directly and stabilize its mRNAs.

As to other genes, they may be regulated by p62/IMP2 directly or indirectly. Here we how the function of cell migration and cell adhesion are important to and relate with cancer metastasis. ADAMTS1 is necessary for normal growth, and ADAMTS1 null mice were observed to manifest growth retardation [87]. In cancer, the role of ADAMTS1 is controversial. This protein is reported to promote tumor growth and metastasis in some cancers while in others its

expression is down-regulated as a tumor suppressor role. It is proposed that the proteolytic status of ADAMTS1 and its mutations determines its impact on tumor metastasis[88]. versican is viewed as an anti-adhesive molecule. A number of recent studies have demonstrated that versican, a large chondroitin sulfate (CS) proteoglycan, harbors anti-adhesive properties and the ability to modulate migration in a number of different cell types, including osteosarcoma cells, smooth muscle cells (SMCs), and various types of tissue fibroblasts [89]. Its anti-adhesive characteristic plays a critical role in cancer. Purified versican is able to reduce attachment of prostate cancer cells and melanoma to fibronectin-coated surfaces in vitro[90], so increased levels of versican in cancer are believed to be associated with progression of cancer to its metastatic disease. Previous research has established that elevated levels of versican are observed in most malignancies such as melanomas, Sarcoma, breast, brain, and ovary cancers [91].

In summary, we identified that p62 is highly expressed in breast cancer tissues. The increased expression of p62/IMP2 can regulate extracellular matrix to increase cell migration and reduce cell adhesion, leading to possible cancer metastasis. We also identified CTGF mRNA as a novel target of p62/IMP2 playing an important role in breast cancer cells. The data further support that p62/IMP2 may also be a target when treating breast cancer metastasis.

7.2 Future Direction

7.2.1 To determine the role of p62/IMP2 in breast cancer metastasis by in vivo study

Our data from in vitro study indicated that p62/IMP2 may be a critical regulator in breast cancer metastasis. In future studies, we will use an in vivo study to confirm if p62/IMP2 really increases the metastatic capacity of breast cancer cells. In regards to which in vivo study, we will use a spontaneous (orthotopic) metastasis assay because this method can enhance the possibility

of distant metastatic spread, compared with ectopic transplantation [92]. p62/IMP2 positive cells and control cells are injected into the mammary fat pad of female SCID mice in two groups respectively (five mice in each group). After inoculation of tumor cells for weeks, the mice will be dissected and examined to see if there is a metastatic spread in the lungs. The metastatic nodules in the lungs will be counted in each group. We will also check to see if metastatic disease will be developed in other viscera. If the result shows that the number of visible metastatic nodules increased significantly per lung in the experimental mice when compared with the control mice, it means that p62/IMP2 expression in breast cancer cells can increase the probability of metastatic colonization in the lungs. We will also measure the average size of metastatic tumor in the lungs of two groups of mice to see if p62/IMP2 will affect the growth rate of metastatic tumor. All metastatic tumors will be photographed. The data mentioned above should be confirmed further by immnohistochemistry staining. All dissected lung tissues will be paraffin-embedded, sectioned, stained with haematoxylin and eosin (H&E) and anti-p62/IMP2 antibody to make sure if tumor tissues are p62/IMP2 positive.

7.2.2 The detection of CTGF and p62/IMP2 expression in the breast cancer cells and the staining of CTGF and p62/IMP2 in tumor tissues.

As a newly identified target of p62/IMP2, CTGF is a very import protein which plays an important role in breast cancer metastasis. In tumors with increased expression of CTGF, tumor angiogenesis and metastasis can be commonly observed. Bone metastasis of breast cancer cells can be inhibited by down-regulating CTGF. Metastasis of breast cancer with high level of CTGF expression was also suppressed by the injected anti-CTGF monoclonal antibodies in vivo. Therefore, it would be very interesting to know how IMP2/p62 regulates CTFG expression in human breast cancer. In our future study, we will detect if p62/IMP2 can increase the CTGF

expression in our variants by Western Blotting. Immunohistochemistry will be also performed to see if a higher expression of CTGF existed in human breast cancer tissues and if there are co-expression of p62/IMP2 with CTGF. Other techniques such SiRNA would be used to see if p62/IMP2 knock-down attenuates expression of CTGF.

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Appendix

List of abbreviations

IMP	IGF2 mRNA binding protein
EMT	Epithelial-mesenchymal transition
ECM	Extracellular matrix
MMP	Metalloproteinases
TAA	Tumor-associated antigen
RIP	RNA immunoprecipitation
VCAN	Versican
CTGF	Connective tissue growth factor
ADAMTS1	ADAM metallopeptidase with thrombospondin type 1 motif, 1
THBS1	Thrombospondin 1
ITGB4	Integrin, beta 4
SDS-PAGE	Sodium docecyle sulfate-polyacrlamide gel eletrophoresis
HRP	Hrseradish peroxidase
IP	Immunoprecipitation
OD	Optical density
PBS	Phosphate-buffered saline

List of Publications and Manuscripts

1) **Li Y,** Zhang Q, Peng B, Shao Q, Qian W, Zhang J.Y. Identification of Glutathione S transferase Omega 1 (GSTO1) protein as a novel tumor-associated antigen and its autoantibody in human esophageal squamous cell carcinoma. Tumor Biol. 2014, 35(11): 10871-7.
2) Shao Q, Ren P, **Li Y**, Peng B, Dai L, Lei N, Yao W, Zhao G, Li L, Zhang J. Y. Autoantibodies against glucose-regulated protein 78 as serological diagnostic biomarkers in hepatocellular carcinoma. Int J Oncol. 2012, 41(3):1061-7.
3) Ren P, Ye H, Dai L, Liu M, Liu X, Chai Y, Shao Q, **Li Y**, Lei N, Peng B, Yao W, Zhang J.Y. Peroxiredoxin 1 is a tumor-associated antigen in esophageal squamous cell carcinoma. Oncol Rep. 2013 30(5):2297-303.
4) Chai Y, Liu X, Dai L, **Li Y**, Liu M, Zhang J.Y. Overexpression of HCC1/CAPERα may play a role in lung cancer carcinogenesis. Tumor Biol. 2014, 35(7):6311-7.
5) Liu X, Peng B, **Li Y**, Lei N, Li W, Zhang J.Y. p90/CIP2A mediates breast cancer cell proliferation and apoptosis. Mol Biol Rep. 2014, 41(11): 7471-8.
6) Liu W, **Li Y**, Wang B, Dai L, Qian W, Zhang J.Y. Autoimmune response to IGF2 mRNAs-binding protein 2 (IMP2/p62) in breast cancer. Scand J Immunol. 2015, 26
7) **Li Y**, Francia G., Zhang, J.Y. The regulation of cell adhesion and cell migration by p62/IMP2 in breast cancer. Oncotarget (Submitted).

Conference Poster Presentation

1) Yang Li, Bo Peng, Ningjing Lei, Giulio Francia, and Jianying Zhang. Overexpression of p62/IMP2 in breast cancer promotes cell migration. American Association for Cancer Research (AACR) Annual Meeting, April 6-10, 2013. Washington, DC, USA.

2) Yang Li, Bo Peng, Ninging Lei, Giulio Francia, and Jianying Zhang. p62/IMP2 promotes breast cancer cell progression. American Association for Cancer Research (AACR) Annual Meeting, April 5-9, 2014. San Diego, CA, USA.

3) Yang Li, Bo Peng, Ningjng Lei, Wei Qian, Giulio Francia, and Jianying Zhang. p62/IMP2 promotes breast cancer progression. DYNAMICA EXPO. November 14-15, 2014. El Paso, TX, USA.

4) Yang Li, Bo Peng, Ningjing Lei, Wei Qian, Giulio Francia, and Jianying Zhang. p62/IMP2 promotes breast cancer metastasis. American Association for Cancer Research (AACR) Annual Meeting. April 18-22, 2015. Philadelphia, PA, USA.

Curriculum Vita

Yang Li was born on October 22nd, 1980 in Zhengzhou, Henan Province, China. She was majored in Biotechnology at Sichuan Agricultural University in China and received her bachelor degree in 2003. Her scientific research began during her studies for the master's degree from 2004 to 2007. When she performed her project "Applications of STR loci in the male Sibling Parentage and Genetic Polymorphism of Y-STR in Han Population in Henan", she developed a strong interest in the field of molecular biology. Therefore after completing her MS degree, she started to pursue her Ph.D. degree at the Department of Biological Sciences, the University of Texas at El Paso in the fall of 2011. She has been working at Dr. Jianying Zhang's lab and her research project focuses on functional study of p62/IMP2 in breast cancer.

During the four years, she revived George A. Krutilek Memorial Graduate Fellowship Fund in 2013 and Frank B. Cotton Trust Graduate Fellowship Fund in 2014. She also received travel awards from Collage of Science in 2013 as well as 2014 for attending American Association for Cancer Research (AACR) Annual Meetings and gave poster presentations in the meetings.

She has published one first-author paper, 5 co-authors papers, and is submitting one first-author manuscript. After graduation, she will apply for a postdoctor training in the field of cancer biology. And her long-term goal is to be a productive scientist and a knowledgeable educator.

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